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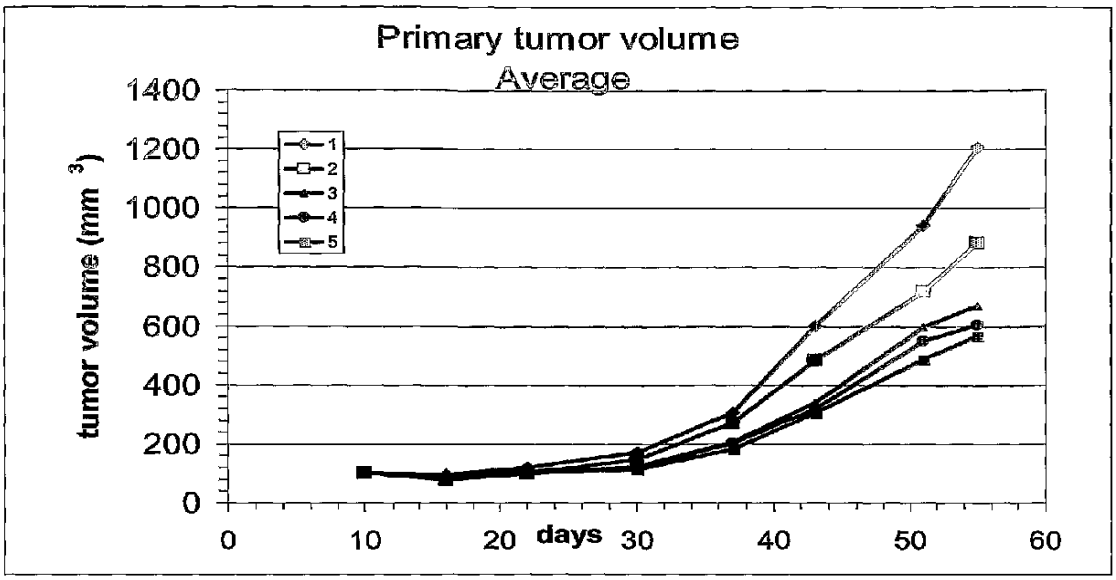
(54) Title: ANTIBODIES AGAINST INSULIN-LIKE GROWTH FACTOR I RECEPTOR AND USES THEREOF



(57) Abstract: An antibody binding to IGF-IR and inhibiting the binding of IGF-I and IGF-II to IG-RI which is characterized in that said antibody is of IgG1 isotype, and shows a ratio of inhibition of the binding of IGF-I to IGF-IR to the inhibition of binding of IGF-II to IGF-IR of 1:3 to 3:1, and induces celle death of 20% or more cells of a preparation of IGF-IR expressing cells after 24 hours at a concentration of said antibody of 100 nM by ADCC; has improved properties in antitumor therapy.

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Antibodies against insulin-like growth factor I receptor and uses thereof

The present invention relates to antibodies against human insulin-like growth factor I receptor (IGF-IR), methods for their production, pharmaceutical compositions containing said antibodies, and uses thereof.

Human insulin-like growth factor I receptor (IGF-IR, EC 2.7.112, CD 221 antigen) belongs to the family of transmembrane protein tyrosine kinases (LeRoith, D., et al., Endocrin. Rev. 16 (1995) 143-163; and Adams, T.E., et al., Cell. Mol. Life Sci. 57 (2000) 1050-1063). IGF-IR binds IGF-I with high affinity and initiates the physiological response to this ligand in vivo. IGF-IR also binds to IGF-II, however with slightly lower affinity. IGF-IR overexpression promotes the neoplastic transformation of cells and there exists evidence that IGF-IR is involved in malignant transformation of cells and is therefore a useful target for the development of therapeutic agents for the treatment of cancer (Adams, T.E., et al., Cell. Mol. Life Sci. 57 (2000) 1050-1063).

Antibodies against IGF-IR are well-known in the state of the art and investigated for their antitumor effects in vitro and in vivo (Benini, S., et al., Clin. Cancer Res. 7 (2001) 1790-1797; Scotlandi, K., et al., Cancer Gene Ther. 9 (2002) 296-307; Scotlandi, K., et al., Int. J. Cancer 101 (2002) 11-16; Brunetti, A., et al., Biochem. Biophys. Res. Commun. 165 (1989) 212-218; Prigent, S.A., et al., J. Biol. Chem. 265 (1990) 9970-9977; Li, S.L., et al., Cancer Immunol. Immunother. 49 (2000) 243-252; Pessino, A., et al., Biochem. Biophys. Res. Commun. 162 (1989) 1236-1243; Surinya, K.H., et al., J. Biol. Chem. 277 (2002) 16718-16725; Soos, M.A., et al., J. Biol. Chem., 267 (1992) 12955-12963; Soos, M.A., et al., Proc. Natl. Acad. Sci. USA 86 (1989) 5217-5221; O'Brien, R.M., et al., EMBO J. 6 (1987) 4003-4010; Taylor, R., et al., Biochem. J. 242 (1987) 123-129; Soos, M.A., et al., Biochem. J. 235 (1986) 199-208; Li, S.L., et al., Biochem. Biophys. Res. Commun. 196 (1993) 92-98; Delafontaine, P., et al., J. Mol. Cell. Cardiol. 26 (1994) 1659-1673; Kull, F.C. Jr., et al. J. Biol. Chem. 258 (1983) 6561-6566; Morgan, D.O., and Roth, R.A., Biochemistry 25 (1986) 1364-1371; Forsayeth, J.R., et al., Proc. Natl. Acad. Sci. USA 84 (1987) 3448-3451; Schaefer, E.M., et al., J. Biol. Chem. 265 (1990) 13248-13253; Gustafson, T.A., and Rutter, W.J., J. Biol. Chem. 265 (1990) 18663-18667; Hoyne, P.A., et al., FEBS Lett. 469 (2000) 57-60; Tulloch, P.A., et al., J. Struct. Biol. 125 (1999) 11-18; Rohlik, Q.T., et al., Biochem. Biophys. Res. Comm. 149 (1987) 276-

281; and Kalebic, T., et al., Cancer Res. 54 (1994) 5531-5534; Adams, T. E., et al., Cell. Mol. Life Sci. 57 (2000) 1050-1063; Dricu, A., et al., Glycobiology 9 (1999) 571-579; Kanter-Lewensohn, L., et al., Melanoma Res. 8 (1998) 389-397; Li, S.L., et al., Cancer Immunol. Immunother. 49 (2000) 243-252). Antibodies against IgF-IR are also described in a lot of further publications, e.g., Arteaga, C.L., et al., Breast Cancer Res. Treatment 22 (1992) 101-106; and Hailey, J., et al., Mol. Cancer Ther. 1 (2002) 1349-1353.

In particular, the monoclonal antibody against IGF-IR called αIR3 is widely used in the investigation of studying IGF-IR mediated processes and IGF-I mediated diseases such as cancer. Alpha-IR-3 was described by Kull, F.C., J. Biol. Chem. 258 (1983) 6561-6566. In the meantime, about a hundred publications have been published dealing with the investigation and therapeutic use of αIR3 in regard to its antitumor effect, alone and together with cytostatic agents such as doxorubicin and vincristine. αIR3 is a murine monoclonal antibody which is known to inhibit IGF-I binding to IGF receptor but not IGF-II binding to IGF-IR. However, there exist other antibodies (e.g., 1H7, Li, S.L., et al., Cancer Immunol. Immunother. 49 (2000) 243-252) which inhibit IGF-II binding to IGF-IR more potently than IGF-I binding. A summary of the state of the art of antibodies and their properties and characteristics is described by Adams, T.E., et al., Cell. Mol. Life Sci. 57 (2000) 1050-1063.

Most of the antibodies described in the state of the art are of mouse origin. Such antibodies are, as is well known in the state of the art, not useful for the therapy of human patients without further alterations like chimerization or humanization. Based on these drawbacks, human antibodies are clearly preferred as therapeutic agents in the treatment of human patients. Human antibodies are well-known in the state of the art (van Dijk, M.A., and van de Winkel, J.G., Curr. Opin. Pharmacol. 5 (2001) 368-374). Based on such technology, human antibodies against a great variety of targets can be produced. Examples of human antibodies against IGF-IR are described in WO 02/053596.

However, there is still a need for antibodies against IGF-IR with convincing benefits for patients in need of antitumor therapy. The relevant benefit for the patient is, in simple terms, reduction in tumor growth and a significant prolongation of time to progression caused by the treatment with the antitumorigenic agent.

Summary of the Invention

The invention comprises an antibody binding to IGF-IR and inhibiting the binding of IGF-I and IGF-II to IGF-IR, characterized in that said antibody is of IgG1 isotype and shows a ratio of inhibition of the binding of IGF-I to IGF-IR to the inhibition of binding of IGF-II to IGF-IR of 1:3 to 3:1 and induces cell death of 20% or more cells of a preparation of IGF-IR expressing cells after 24 hours at a concentration of said antibody of 100 nM by ADCC.

Antibodies according to the invention show benefits for patients in need of antitumor therapy and provide reduction of tumor growth and a significant prolongation of the time to progression. The antibodies according to the invention have new and inventive properties causing a benefit for a patient suffering from a disease associated with an IGF deregulation, especially a tumor disease. The antibodies according to the invention are characterized by the abovementioned properties. The properties are therefore especially specific binding to IGF-IR, inhibiting the binding of IGF-I and IGF-II to IGF-IR at the abovementioned ratio, being of IgG1 isotype, and having effector function in ADCC.

Preferably, in addition, the antibodies according to the invention induce cell death of 20% or more cells of a preparation of IGF-IR expressing cells after 4 h at an antibody concentration of 100 nM by CDC.

Preferably, at a concentration of 50 nM the antibodies according to the invention completely inhibit IGF-I mediated signal transduction of IGF-IR in tumor cells.

The invention also comprises antibody encoding nucleic acids. The encoded polypeptides are capable of assembling together with the respective other antibody chain defined below:

- an antibody heavy chain comprising as CDRs CDR1 (aa 31-35), CDR2 (aa 50-66) and CDR3 (aa 98-108) of SEQ ID NO:1, wherein amino acid 31 can be asparagine or serine, amino acid 66 can be glycine or deleted, and amino acid 104 can be glutamic acid or aspartic acid;

- an antibody light chain comprising as CDRs CDR1 (aa 18-34 or aa 24-34), CDR2 (aa 50-56) and CDR3 (aa 89-98) of SEQ ID NO:2, wherein amino acid 96 can be proline or isoleucine, and amino acid 98 can be phenylalanine or deleted.

The preferred CDRs are (a) CDR1 (aa 31-35), CDR2 (aa 50-65) and CDR3 (aa 98-108) of SEQ ID NO:1, wherein amino acid 31 can be asparagine or serine and amino acid 104 can be glutamic acid or asparatic acid, and (b) CDR1 (aa 24-34), CDR2 (aa 50-56) and CDR3 (aa 89-97) of SEQ ID NO:2.

CDR numbering and definition is preferred according to Kabat, E. (see e.g. Johnson, G., et al., Nucl. Acids Res. 28 (2000) 214-218).

Preferably, the nucleic acid encodes a polypeptide which is either a heavy chain consisting of a variable region (VH) of SEQ ID NO:1, wherein amino acid (aa) 30 denotes serine or arginine, aa 31 denotes asparagine or serine, aa 94 denotes histidine or tyrosine and aa 104 denotes aspartic acid or glutamic acid, and of a human heavy chain constant region (CH);
and a light chain consisting of a variable region (VL) of SEQ ID NO:2, wherein aa 96 denotes proline or isoleucine, aa 100 denotes proline or glutamine, aa 103 denotes arginine or lysine, aa 104 denotes valine or leucine and aa 105 denotes aspartic acid or glutamic acid, and of a human light chain constant region (CL).

The antibody is preferably a monoclonal antibody and, in addition, a chimeric antibody (human constant chain), a humanized antibody and especially preferably a human antibody.

The antibody binds to IGF-IR human (EC 2.7.1.112, SwissProt P08069) in competition to the antibodies characterized by the variable chains of SEQ ID NOS:1-6.

The antibody is further characterized by an affinity of 10^{-8} M (K_D) or less, preferably of about 10^{-8} to 10^{-11} M.

Preferably, the invention provides antibodies comprising as complementarity determining regions (CDRs) having the following sequences:

- an antibody heavy chain comprising as CDRs CDR1 (aa 31-35), CDR2 (aa 50-66) and CDR3 (aa 98-108) of SEQ ID NO:1, wherein amino acid 31 can be asparagine or serine, amino acid 66 can be glycine or deleted, and amino acid 104 can be glutamic acid or aspartic acid;
- an antibody light chain comprising as CDRs CDR1 (aa 18-34 or aa 24-34), CDR2 (aa 50-56) and CDR3 (aa 89-98) of SEQ ID NO:2, wherein amino acid 96 can be proline or isoleucine, and amino acid 98 can be phenylalanine or deleted.

The invention therefore comprises also a polypeptide and an encoding nucleic acid selected from the above-mentioned group consisting of CDR1, CDR2, CDR3 of heavy chain and CDR1, CDR2, CDR3 of light chain of an IGF-IR antibody according to the invention.

Preferably, the invention comprises an antibody characterized by a heavy chain consisting of a variable region (VH) of SEQ ID NO:1, wherein amino acid (aa) 30 denotes serine or arginine, aa 31 denotes asparagine or serine, aa 94 denotes histidine or tyrosine and aa 104 denotes aspartic acid or glutamic acid, and of a human heavy chain constant region (CH);
and a light chain consisting of a variable region (VL) of SEQ ID NO:2, wherein aa 96 denotes proline or isoleucine, aa 100 denotes proline or glutamine, aa 103 denotes arginine or lysine, aa 104 denotes valine or leucine and aa 105 denotes aspartic acid or glutamic acid, and of a human light chain constant region (CL).

The constant regions provide C1q complement binding and are therefore preferably of human IgG1 type.

The combinations
aa 30 Arg, aa 31 Asn, aa 94 Tyr and aa 104 Asp (antibody 1A) or
aa 30 Arg, aa 31 Ser, aa 94 Tyr and aa 104 Asp (antibody 8) or
aa 30 Ser, aa 31 Asn, aa 94 His and aa 104 Glu (antibody 23)
in the heavy chain are preferred.

The combinations
aa 96 Pro, aa 100 Pro, aa 103 Lys, aa 104 Val and aa 105 Asp (antibody 1A and 8),
aa 96 Ile, aa 100 Gln, aa 103 Arg, aa 104 Leu and aa 105 Glu (antibody 23)
in the light chain are especially preferred.

The combination aa 30 Arg, aa 31 Asn, aa 94 Tyr, and aa 104 Asp in the heavy chain and aa 96 Pro, aa 100 Pro, aa 103 Lys, aa 104 Val and aa 105 Asp in the light chain is especially preferred.

The antibody according to the invention considerably prolongates the time to progression in relevant xenograft tumor models in comparison with vehicle treated animals and reduces tumor growth. The antibody inhibits the binding of IGF-I and IGF-II to IGF-IR in vitro and in vivo, preferably in about an equal manner for IGF-I and IGF-II.

The antibody is further characterized by the ability to bind IgGFc receptor and to induce ADCC and preferably to bind complement component C1q and to induce CDC.

The invention further provides hybridoma cell lines which produce such antagonistic monoclonal antibodies according to the invention.

The preferred hybridoma cell lines according to the invention, <IGF-1R> HuMab Clone 1a (antibody 1A, Ab 1A or Ak 1A), <IGF-1R> HuMab Clone 23 (antibody 23), and <IGF-1R> HuMab-Clone 8 (antibody 8) were deposited with Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany:

Cell line	Deposition No.	Date of Deposit
<IGF-1R> HUMAB Clone 1a	DSM ACC 2586	10.04.2003
<IGF-1R> HUMAB Clone 23	DSM ACC 2588	10.04.2003
<IGF-1R> HUMAB-Clone 8	DSM ACC 2589	24.04.2003

The antibodies obtainable from said cell lines are preferred embodiments of the invention.

The invention further provides nucleic acids encoding such antibodies, expression vectors containing said nucleic acids, and host cells for the recombinant production of such antibodies.

The invention further provides methods for the recombinant production of such antibodies.

The invention further provides methods for treating cancer, comprising administering to a patient diagnosed as having cancer (and therefore being in need of an antitumor therapy) an effective amount of an antagonistic antibody against IGF-IR according to the invention. The antibody may be administered alone, in a pharmaceutical composition, or alternatively in combination with a cytotoxic treatment such as radiotherapy or a cytotoxic agent or a prodrug thereof.

The invention further comprises the use of an antibody according to the invention for cancer treatment and for the manufacture of a pharmaceutical composition according to the invention. In addition, the invention comprises a method for the manufacture of a pharmaceutical composition according to the invention.

The invention further comprises a pharmaceutical composition containing an antibody according to the invention with a pharmaceutically effective amount, optionally together with a buffer and/or an adjuvant useful for the formulation of antibodies for pharmaceutical purposes.

The invention further provides a pharmaceutical composition comprising such an antibody in a pharmaceutically acceptable carrier. In one embodiment, the pharmaceutical composition may be included in an article of manufacture or kit.

The invention further comprises a vector containing a nucleic acid according to the invention, capable of expressing said nucleic acid in a prokaryotic or eukaryotic host cell.

The invention further comprises a prokaryotic or eukaryotic host cell comprising a vector according to the invention.

The invention further comprises a method for the production of a recombinant human antibody according to the invention, characterized by expressing a nucleic acid according to the invention in a prokaryotic or eukaryotic host cell and recovering said antibody from said cell. The invention further comprises the antibody obtainable by such a recombinant method.

Detailed Description of the Invention

The term "antibody" encompasses the various forms of antibodies including but not being limited to whole antibodies, antibody fragments, human antibodies, humanized antibodies and genetically engineered antibodies as long as the characteristic properties according to the invention are retained.

"Antibody fragments" comprise a portion of a full length antibody, generally at least the antigen binding portion or the variable region thereof. Examples of antibody fragments include diabodies, single-chain antibody molecules, immunotoxins, and multispecific antibodies formed from antibody fragments. In addition, antibody fragments comprise single chain polypeptides having the characteristics of a VH chain, namely being able to assemble together with a VL chain or of a VL chain binding to IGF-1R, namely being able to assemble together with a VH chain to a functional antigen binding pocket and thereby providing the property of inhibiting the binding of IGF-I and IGF-II to IGF-IR.

"Antibody fragments" also comprises such fragments which per se are not able to provide effector functions (ADCC/CDC) but provide this function in a manner according to the invention after being combined with appropriate antibody constant domain(s).

The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of a single amino acid composition. Accordingly, the term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable and constant regions derived from human germline immunoglobulin sequences. In one embodiment, the human monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic non-human animal, e.g. a transgenic mouse, having a genome comprising a human heavy chain transgene and a light human chain transgene fused to an immortalized cell.

The term "chimeric antibody" refers to a monoclonal antibody comprising a variable region, i.e., binding region, from one source or species and at least a portion of a constant region derived from a different source or species, usually prepared by recombinant DNA techniques. Chimeric antibodies comprising a

murine variable region and a human constant region are especially preferred. Such murine/human chimeric antibodies are the product of expressed immunoglobulin genes comprising DNA segments encoding murine immunoglobulin variable regions and DNA segments encoding human immunoglobulin constant regions. Other forms of "chimeric antibodies" encompassed by the present invention are those in which the class or subclass has been modified or changed from that of the original antibody. Such "chimeric" antibodies are also referred to as "class-switched antibodies." Methods for producing chimeric antibodies involve conventional recombinant DNA and gene transfection techniques now well known in the art. See, e.g., Morrison, S.L., et al., Proc. Natl. Acad Sci. USA 81 (1984) 6851-6855; US Patent Nos. 5,202,238 and 5,204,244.

The term "humanized antibody" refers to antibodies in which the framework or "complementarity determining regions" (CDR) have been modified to comprise the CDR of an immunoglobulin of different specificity as compared to that of the parent immunoglobulin. In a preferred embodiment, a murine CDR is grafted into the framework region of a human antibody to prepare the "humanized antibody." See, e.g., Riechmann, L., et al., Nature 332 (1988) 323-327; and Neuberger, M.S., et al., Nature 314 (1985) 268-270. Particularly preferred CDRs correspond to those representing sequences recognizing the antigens noted above for chimeric and bifunctional antibodies.

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The variable heavy chain is preferably derived from germline sequence DP-61 (GenBank M99682) and the variable light chain is preferably derived from germline sequence L15 (GenBank K01323). The constant regions of the antibody are constant regions of human IgG1 type. Such regions can be allotypic and are described by, e.g., Johnson, G., and Wu, T.T., Nucleic Acids Res. 28 (2000) 214-218 and the databases referenced therein and are useful as long as the properties of induction of ADCC and preferably CDC according to the invention are retained.

The term "recombinant human antibody", as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies isolated from a host cell such as a NS0 or CHO cell or

from an animal (e.g. a mouse) that is transgenic for human immunoglobulin genes or antibodies expressed using a recombinant expression vector transfected into a host cell. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences in a rearranged form. The recombinant human antibodies according to the invention have been subjected to in vivo somatic hypermutation. Thus, the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire in vivo.

As used herein, " binding" refers to antibody binding to IGF-IR with an affinity of about 10^{-11} to 10^{-8} M (K_D), preferably of about 10^{-11} to 10^{-9} M.

The term "nucleic acid molecule", as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The "constant domains" are not involved directly in binding the antibody to an antigen but are involved in the effector functions (ADCC, complement binding, and CDC). The constant domain of an antibody according to the invention is therefore preferably of the human IgG1 type. Human constant domains having these characteristics are described in detail by Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991), and by Brüggemann, M., et al., J. Exp. Med. 166 (1987) 1351-1361; Love, T.W., et al., Methods Enzymol. 178 (1989) 515-527. Examples are shown in SEQ ID NOS:7 to 10. Other useful constant domains are the constant domains of the antibodies obtainable from the hybridoma cell lines deposited with DSMZ for this invention. The constant domains useful in the invention provide complement binding. ADCC and optionally CDC are provided by the combination of variable and constant domains.

The "variable region" (variable region of a light chain (VL), variable region of a heavy chain (VH)) as used herein denotes each of the pair of light and heavy chains which is involved directly in binding the antibody to the antigen. The domains of variable human light and heavy chains have the same general structure and each domain comprises four framework (FR) regions whose sequences are widely

conserved, connected by three "hypervariable regions" (or complementarity determining regions, CDRs). The framework regions adopt a β-sheet conformation and the CDRs may form loops connecting the β-sheet structure. The CDRs in each chain are held in their three-dimensional structure by the framework regions and form together with the CDRs from the other chain the antigen binding site. The antibody heavy and light chain CDR3 regions play a particularly important role in the binding specificity/affinity of the antibodies according to the invention and therefore provide a further object of the invention.

The terms "hypervariable region" or "antigen-binding portion of an antibody" when used herein refer to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from the "complementarity determining regions" or "CDRs". "Framework" or "FR" regions are those variable domain regions other than the hypervariable region residues as herein defined. Therefore, the light and heavy chains of an antibody comprise from N- to C-terminus the domains FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. Especially, CDR3 of the heavy chain is the region which contributes most to antigen binding. CDR and FR regions are determined according to the standard definition of Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop".

The term "binding to IGF-IR" as used herein means the binding of the antibody to IGF-IR in an in vitro assay, preferably in a binding assay in which the antibody is bound to a surface and binding of IGF-IR is measured by Surface Plasmon Resonance (SPR). Binding means a binding affinity (K_D) of 10^{-8} M or less, preferably 10^{-11} to 10^{-8} M.

Binding to IGF-IR can be investigated by a BIAcore assay (Pharmacia Biosensor AB, Uppsala, Sweden). The affinity of the binding is defined by the terms ka (rate constant for the association of the antibody from the antibody/antigen complex, kd (dissociation constant), and K_D (kd/ka). The antibodies according to the invention preferably show a K_D of 10^{-9} M or less.

The binding of IGF-I and IGF-II to IGF-IR is also inhibited by the antibodies according to the invention. The inhibition is measured as IC_{50} in an assay for

binding of IGF-I/IGF-II to IGF-IR on tumor cells. Such an assay is described in Example 7. In such an assay, the amount of radiolabeled IGF-I or IGF-II or IGF-IR binding fragments thereof bound to the IGF-IR provided at the surface of said tumor cells (e.g. HT29) is measured without and with increasing concentrations of the antibody. The IC_{50} values of the antibodies according to the invention for the binding of IGF-I and IGF-II to IGF-IR are no more than 10 nM and the ratio of the IC_{50} values for binding of IGF-I/IGF-II to IGF-IR is about 1:3 to 3:1.

The term "inhibiting the binding of IGF-I and IGF-II to IGF-IR" as used herein refers to inhibiting the binding of I^{125}-labeled IGF-I or IGF-II to IGF-IR presented on the surface of HT29 (ATCC HTB-38) tumor cells in an in vitro assay. Inhibiting means an IC_{50} value of 10 nM or lower.

The term "IGF-IR expressing cells" refers to such cells which are overexpressing IGF-I receptor to about at least 20,000 receptors/cell. Such cells are, for example, tumor cell lines such as NCI H322M, or a cell line (e.g. 3T3) overexpressing IGF-IR after transfection with an expression vector for IGF-IR.

The term "antibody-dependent cellular cytotoxicity (ADCC)" refers to lysis of human tumor target cells by an antibody according to the invention in the presence of effector cells. ADCC is measured preferably by the treatment of a preparation of IGF-IR expressing cells with an antibody according to the invention in the presence of effector cells such as freshly isolated PBMC or purified effector cells from buffy coats, like monocytes or NK cells. ADCC is found if the antibody induces at a concentration of 100 nM the lysis (cell death) of 20% or more of the tumor cells after 24 hours. The assay is performed preferably with ^{51}Cr labeled tumor cells and measurement of specifically released ^{51}Cr. Controls include the incubation of the tumor target cells with effector cells but without the antibody.

The term "complement-dependent cytotoxicity (CDC)" refers to lysis of human tumor target cells by the antibody according to the invention in the presence of complement. CDC is measured preferably by the treatment of a preparation of IGF-IR expressing cells with an antibody according to the invention in the presence of complement. CDC is found if the antibody induces at a concentration of 100 nM the lysis (cell death) of 20% or more of the tumor cells after 4 hours. The assay is performed preferably with ^{51}Cr labeled tumor cells and measurement of released

^{51}Cr. Controls include the incubation of the tumor target cells with complement but without the antibody.

The term "complete inhibition of IGF-I mediated signal transduction" refers to the inhibition of IGF-I-mediated phosphorylation of IGF-IR. For such an assay, IGF-IR expressing cells, preferably H322M cells, are stimulated with IGF-I and treated with an antibody according to the invention (an antibody concentration of 10 nM or lower (IC_{50}) is useful). Subsequently, an SDS PAGE is performed and phosphorylation of IGF-I is measured by Western blotting analysis with an antibody specific for phosphorylated tyrosine. Complete inhibition of the signal transduction is found if on the Western blot visibly no band can be detected which refers to phosphorylated IGF-IR.

The antibodies according to the invention show a binding to the same epitope of IGF-IR as antibody 1A or are inhibited in binding to IGF-IR due to steric hindrance of binding by antibody 1A. Binding inhibition can be detected by an SPR assay using immobilized antibody 1A and IGF-IR at a concentration of 20-50 nM and the antibody to be detected at a concentration of 100 nM. A signal reduction of 50% or more shows that the antibody competes with antibody 1A. Such an assay can be performed in the same manner by using antibody 8 or 23 as immobilized antibodies.

The term "epitope" means a protein determinant capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

The antibodies according to the invention include, in addition, such antibodies having "conservative sequence modifications", nucleotide and amino acid sequence modifications which do not affect or alter the above-mentioned characteristics of the antibody according to the invention. Modifications can be introduced by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions include ones in

which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g. glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a human anti-IGF-IR antibody can be preferably replaced with another amino acid residue from the same side chain family.

Amino acid substitutions can be performed by mutagenesis based upon molecular modeling as described by Riechmann, L., et al., Nature 332 (1988) 323-327 and Queen, C., et al., Proc. Natl. Acad. Sci. USA 86 (1989)10029-10033.

In a preferred embodiment of the invention, the antibodies according to the invention are further characterized by one or more of the characteristics selected from the group selected from the binding parameters ka, kd and K_D, binding to the same epitope to which antibodies 1A, 8 and 23 bind, the IC_{50} values for inhibition of binding of IGF-I and IGF-II to IGF-IR on tumor cells, and the IC_{50} values for inhibition of phosphorylation of IGF-IR upon stimulation of IGF-I in tumor cells. Inhibition of phosphorylation of IGF-IR leads to the inhibition of phosphorylation of downstream elements such as PkB, the down-regulation of IGF-IR in tumor cells, and the influence on the three-dimensional growth of tumor cells in vitro. The antibodies are further preferably characterized by their pharmacokinetic and pharmacodynamic values, and the cross-reactivity for other species.

The antibodies according to the invention preferably inhibit IGF-IR tyrosine phosphorylation.

The antibodies according to the invention preferably downregulate the IGF-IR protein level in tumor cells.

The antibodies according to the invention inhibit preferably the three-dimensional growth of tumor cells in a colony formation assay as well as proliferation of IGF-IR expressing cells (e.g. NIH 3T3 cells).

The antibodies according to the invention preferably show cross-reactivity with IGF-IR from Marmoset (Callithrix jacchus) and Cynomolgus (Macaca fascicularis), but not with IGF-IR from rat and mouse. After two weeks' treatment of healthy Macaca fasciularis primates, no signs of side-effects could be detected (200 mg/kg/week).

The antibodies according to the invention preferably do not inhibit binding of insulin to insulin receptor in a binding competition assay on insulin receptor overexpressing 3T3 cells using the antibody in a concentration of 200 nmol/l or more.

The antibodies according to the invention preferably show serum half-lives of about 10-18 days in vivo (in nude mice such as NMRI mice).

The antibodies according to the invention are preferably produced by recombinant means. Such methods are widely known in the state of the art and comprise protein expression in prokaryotic and eukaryotic cells with subsequent isolation of the antibody polypeptide and usually purification to a pharmaceutically acceptable purity. For the protein expression, nucleic acids encoding light and heavy chains or fragments thereof are inserted into expression vectors by standard methods. Expression is performed in appropriate prokaryotic or eukaryotic host cells like CHO cells, NS0 cells, SP2/0 cells, HEK293 cells, COS cells, yeast, or E.coli cells, and the antibody is recovered from the cells (supernatant or cells after lysis).

Recombinant production of antibodies is well-known in the state of the art and described, for example, in the review articles of Makrides, S.C., Protein Expr. Purif. 17 (1999) 183-202; Geisse, S., et al., Protein Expr. Purif. 8 (1996) 271-282; Kaufman, R.J., Mol. Biotechnol. 16 (2000) 151-161; Werner, R.G., Drug Res. 48 (1998) 870-880.

The antibodies may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form. Purification is performed in order to eliminate

other cellular components or other contaminants, e.g. other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis, and others well known in the art. See Ausubel, F., et al., ed. Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York (1987).

Expression in NS0 cells is described by, e.g., Barnes, L.M., et al., Cytotechnology 32 (2000) 109-123; and Barnes, L.M., et al., Biotech. Bioeng. 73 (2001) 261-270. Transient expression is described by, e.g., Durocher, Y., et al., Nucl. Acids. Res. 30 (2002) E9. Cloning of variable domains is described by Orlandi, R., et al., Proc. Natl. Acad. Sci. USA 86 (1989) 3833-3837; Carter, P., et al., Proc. Natl. Acad. Sci. USA 89 (1992) 4285-4289; and Norderhaug, L., et al., J. Immunol. Methods 204 (1997) 77-87. A preferred transient expression system (HEK 293) is described by Schlaeger, E.-J., and Christensen, K., in Cytotechnology 30 (1999) 71-83 and by Schlaeger, E.-J., in J. Immunol. Methods 194 (1996) 191-199.

The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, enhancers and polyadenylation signals.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading frame. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The monoclonal antibodies are suitably separated from the culture medium by conventional immunoglobulin purification procedures such as, for example,

protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. DNA and RNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures. The hybridoma cells can serve as a source of such DNA and RNA. Once isolated, the DNA may be inserted into expression vectors, which are then transfected into host cells such as HEK 293 cells, CHO cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of recombinant monoclonal antibodies in the host cells.

Amino acid sequence variants of human IGF-IR antibody are prepared by introducing appropriate nucleotide changes into the antibody DNA, or by peptide synthesis. Such modifications can be performed, however, only in a very limited range, e.g. as described above. For example, the modifications do not alter the abovementioned antibody characteristics such as the IgG isotype and epitope binding, but may improve the yield of the recombinant production, protein stability or facilitate the purification.

Any cysteine residue not involved in maintaining the proper conformation of the anti- IGF-IR antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody. Glycosylation of antibodies is typically N-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites).

Nucleic acid molecules encoding amino acid sequence variants of anti-IGF-IR antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of humanized anti-IGF-IR antibody.

The invention also pertains to immunoconjugates comprising the antibody according to the invention conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g., an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), a radioactive isotope (i.e., a radioconjugate) or a prodrug of a cytotoxic agent. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleuritesfordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters; (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p- azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediatnine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta, E.S., et al., Science 238 (1987) 1098-1104). Carbon- 14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO 94/11026.

Another type of covalent modification involves chemically or enzymatically coupling glycosides to the antibody. These procedures are advantageous in that they

do not require production of the antibody in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330, and in Aplin, J.D., and Wriston, J.C. Jr., CRC Crit. Rev. Biochem. (1981) 259-306.

Removal of any carbohydrate moieties present on the antibody may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the antibody to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N- acetylgalactosamine), while leaving the antibody intact. Chemical deglycosylation is described by Sojahr, H.T., and Bahl, O.P., Arch. Biochem. Biophys. 259 (1987) 52-57 and by Edge, A.S., et al. Anal. Biochem. 118 (1981) 131-137. Enzymatic cleavage of carbohydrate moieties on antibodies can be achieved by the use of a variety of endo- and exo- glycosidases as described by Thotakura, N.R., and Bahl, O.P., Meth. Enzymol. 138 (1987) 350-359.

Another type of covalent modification of the antibody comprises linking the antibody to one of a variety of nonproteinaceous polymers, eg., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in US Patent Nos. 4,640,835; 4,496,689; 4,301, 144; 4,670,417; 4,791,192 or 4,179,337.

In yet another aspect, the invention provides isolated B-cells from a transgenic non-human animal, e.g. a transgenic mouse, which express the human anti IGF-IR antibodies according to the invention. Preferably, the isolated B cells are obtained from a transgenic non-human animal, e.g., a transgenic mouse, which has been immunized with a purified or enriched preparation of IGF-IR antigen and/or cells expressing IGF-IR. Preferably, the transgenic non-human animal, e.g. a transgenic mouse, has a genome comprising a human heavy chain transgene and a human light chain transgene encoding all or a portion of an antibody of the invention. The isolated B-cells are then immortalized to provide a source (e.g. a hybridoma) of human anti-IGF-IR antibodies. Accordingly, the present invention also provides a

hybridoma capable of producing human monoclonal antibodies according to the invention. In one embodiment, the hybridoma includes a B cell obtained from a transgenic non-human animal, e.g., a transgenic mouse having a genome comprising a human heavy chain transgene and a human light chain transgene encoding all or a portion of an antibody of the invention, fused to an immortalized cell.

In a particular embodiment, the transgenic non-human animal is a transgenic mouse having a genome comprising a human heavy chain transgene and a human light chain transgene encoding all or a portion of an antibody of the invention. The transgenic non-human animal can be immunized with a purified or enriched preparation of IGF-IR antigen and/or cells expressing IGF-IR. Preferably, the transgenic non-human animal, e.g. the transgenic mouse, is capable of producing IgG1 isotypes of human monoclonal antibodies to IGF-IR.

The human monoclonal antibodies according to the invention can be produced by immunizing a transgenic non-human animal, e.g. a transgenic mouse, having a genome comprising a human heavy chain transgene and a human light chain transgene encoding all or a portion of an antibody of the invention, with a purified or enriched preparation of IGF-IR antigen and/or cells expressing IGF-IR. B cells (e.g. splenic B cells) of the animal are then obtained and fused with myeloma cells to form immortal, hybridoma cells that secrete human monoclonal antibodies against IGF-IR.

In a preferred embodiment, human monoclonal antibodies directed against IGF-IR can be generated using transgenic mice carrying parts of the human immune system rather than the mouse system. These transgenic mice, referred to herein as "HuMab" mice, contain a human immunoglobulin gene miniloci that encodes unrearranged human immunoglobulin genes which include the heavy (μ and γ) and κ light chain (constant region genes), together with targeted mutations that inactivate the endogenous μ and κ chain loci (Lonberg, N., et al., Nature 368 (1994) 856-859). Accordingly, the mice exhibit reduced expression of mouse IgM or κ, and in response to immunization, the introduced human heavy and light chain transgenes undergo class switching and somatic mutation to generate high affinity human IgG monoclonal antibodies (Lonberg, N., et al., Nature 368 (1994) 856-859; reviewed in Lonberg, N., Handbook of Experimental Pharmacology 113 (1994) 49-

101; Lonberg, N., and Huszar, D., Intern. Rev. Immunol. 25 (1995) 65-93; and Harding, F., and Lonberg, N., Ann. N. Acad. Sci 764 (1995) 536-546). The preparation of HuMab mice is described in Taylor, L., et al., Nucleic Acids Research 20 (1992) 6287-6295; Chen, J., et al., International Immunology 5 (1993) 647-656; Tuaillon, N., et al., Proc. Natl. Acad. Sci USA 90 (1993) 3720-3724; Choi, T.K., et al., Nature Genetics 4 (1993) 117-123; Chen, J., et al., EMBO J. 12 (1993) 821-830; Tuaillon, N., et al., Immunol. 152 (1994) 2912-2920; Lonberg, N., et al., Nature 368 (1994) 856-859; Lonberg, N., Handbook of Experimental Pharmacology 113 (1994) 49-101; Taylor, L., et al., Int. Immunol. 6 (1994) 579-591; Lonberg, N., and Huszar, D., Intern. Rev. Immunol. 25 (1995) 65-93; Harding, F., and Lonberg, N., Ann. N. Acad. Sci 764 (1995) 536-546; Fishwild, D.M., et al., Nat. Biotechnol. 14 (1996) 845-851, the contents of all of which are hereby incorporated by reference in their entirety. See further, US Patent Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877, 397; 5,661,016; 5,814,318; 5,874,299; 5,545,807; 5,770,429; WO 98/24884; WO 94/25585; WO 93/1227; WO 92/22645; and WO 92/03918.

To generate fully human monoclonal antibodies to IGF-IR, HuMab mice can be immunized with a purified or enriched preparation of IGF-IR antigen and/or cells expressing IGF-IR in accordance with the general method, as described by Lonberg, N., et al., Nature 368 (1994) 856-859; Fishwild, D.M., et al., Nat. Biotechnol. 14 (1996) 845-851 and WO 98/24884. Preferably, the mice will be 6-16 weeks of age upon the first immunization. For example, a purified or enriched preparation of soluble IGF-IR antigen (e.g. purified from IGF-IR-expressing cells) can be used to immunize the HuMab mice intraperitoneally. In the event that immunizations using a purified or enriched preparation of IGF-IR antigen do not result in antibodies, mice can also be immunized with cells expressing IGF-IR, e.g., a tumor cell line, to promote immune responses. Cumulative experience with various antigens has shown that the HuMab transgenic mice respond best when initially immunized intraperitoneally (i.p.) with antigen in complete Freund's adjuvant, followed by every other week i.p. immunizations (for example, up to a total of 6) with antigen in incomplete Freund's adjuvant. The immune response can be monitored over the course of the immunization protocol with plasma samples being obtained by retroorbital bleeds. The plasma can be screened by ELISA , and mice with sufficient titers of anti-IGF-IR human immunoglobulin can be used for immortalization of corresponding B cells. Mice can be boosted intravenously with antigen 3 to 4 days before sacrifice and removal of the spleen and lymph nodes. It is

expected that 2-3 fusions for each antigen may need to be performed. Several mice will be immunized for each antigen. For example, a total of twelve HuMab mice of the HCo7 and HCo12 strains can be immunized.

The HCo7 mice have a JKD disruption in their endogenous light chain (kappa) genes (as described in Chen, J., et al., EMBO J. 12 (1993) 821-830), a CMD disruption in their endogenous heavy chain genes (as described in Example 1 of WO 01/14424), a KCo5 human kappa light chain transgene (as described in Fishwild, D.M., et al., Nat. Biotechnol. 14 (1996) 845-851), and a HCo7 human heavy chain transgene (as described in US Patent No. 5,770,429).

The HCo12 mice have a JKD disruption in their endogenous light chain (kappa) genes (as described in Chen, J., et al., EMBO J. 12 (1993) 821-830), a CMD disruption in their endogenous heavy chain genes (as described in Example 1 of WO 01/14424), a KCo5 human kappa light chain transgene (as described in Fishwild, D.M., et al., Nat. Biotechnol. 14 (1996) 845-851), and a HCo12 human heavy chain transgene (as described in Example 2 of WO 01/14424).

The mouse lymphocytes can be isolated and fused with a mouse myeloma cell line using PEG based on standard protocols to generate hybridomas. The resulting hybridomas are then screened for the production of antigen-specific antibodies. For example, single cell suspensions of splenic and lymph node-derived lymphocytes from immunized mice are fused to one-sixth the number of SP 2/0 nonsecreting mouse myeloma cells (ATCC, CRL 1581) with 50% PEG. Cells are plated at approximately 2 x 10^5 in flat bottom microtiter plate, followed by about two weeks incubation in selective medium.

Individual wells are then screened by ELISA for human anti-IGF-IR monoclonal IgM and IgG antibodies. Once extensive hybridoma growth occurs, medium is analyzed, usually after 10-14 days. The antibody secreting hybridomas are replated, screened again, and if still positive for human IgG, anti-IGF-IR monoclonal antibodies, can be subcloned at least twice by limiting dilution. The stable subclones are then cultured in vitro to produce antibody in tissue culture medium for characterization.

Because CDR sequences are responsible for antibody-antigen interactions, it is possible to express recombinant antibodies according to the invention by constructing expression vectors that include the CDR sequences according to the invention onto framework sequences from a different human antibody (see, e.g., Riechmann, L., et al., Nature 332 (1998) 323-327; Jones, P., et al., Nature 321 (1986) 522-525; and Queen, C., et al., Proc. Natl. Acad. See. U.S.A. 86 (1989)10029-10033). Such framework sequences can be obtained from public DNA databases that include germline human antibody gene sequences. These germline sequences will differ from mature antibody gene sequences because they will not include completely assembled variable genes, which are formed by V(D)J joining during B cell maturation. Germline gene sequences will also differ from the sequences of a high affinity secondary repertoire antibody at individual evenly across the variable region.

The invention preferably comprises a nucleic acid fragment encoding a polypeptide binding to IGF-IR, whereby said polypeptide inhibits the binding of IGF-I and IGF-II to IGF-IR, selected from the group consisting of

a heavy chain consisting of a variable region (VH) of SEQ ID NO:1, wherein amino acid (aa) 30 denotes serine or arginine, aa 31 denotes asparagine or serine, aa 94 denotes histidine or tyrosine and aa 104 denotes aspartic acid or glutamic acid, and of a human heavy chain constant region (CH) or a fragment thereof;
and a light chain consisting of a variable region (VL) of SEQ ID NO:2, wherein aa 96 denotes proline or isoleucine, aa 100 denotes proline or glutamine, aa 103 denotes arginine or lysine, aa 104 denotes valine or leucine and aa 105 denotes aspartic acid or glutamic acid, and of a human light chain constant region (CL) or a fragment thereof.

Particularly preferred nucleic acid fragments according to the invention are nucleic acid fragments encoding a polypeptide according to the invention comprising as CDR regions

an antibody heavy chain comprising as CDRs CDR1 (aa 31-35), CDR2 (aa 50-66) and CDR3 (aa 98-108) of SEQ ID NO:1, wherein amino acid 31 can be asparagine or serine, amino acid 66 can be glycine or deleted, and amino acid 104 can be glutamic acid or aspartic acid;

an antibody light chain comprising as CDRs CDR1 (aa 18-34 or aa 24-34), CDR2 (aa 50-56) and CDR3 (aa 89-98) of SEQ ID NO:2, wherein amino acid 96 can be proline or isoleucine, and amino acid 98 can be phenylalanine or deleted.

The reconstructed heavy and light chain variable regions are combined with sequences of promoter, translation initiation, constant region, 3' untranslated, polyadenylation, and transcription termination to form expression vector constructs. The heavy and light chain expression constructs can be combined into a single vector, co-transfected, serially transfected, or separately transfected into host cells which are then fused to form a single host cell expressing both chains.

Accordingly, the invention provides a method for the production of a recombinant human antibody according to the invention, characterized by expressing a nucleic acid encoding

a heavy chain consisting of a variable region (VH) of SEQ ID NO:1, wherein amino acid (aa) 30 denotes serine or arginine, aa 31 denotes asparagine or serine, aa 94 denotes histidine or tyrosine and aa 104 denotes aspartic acid or glutamic acid, and of a human heavy chain constant region (CH);
and a light chain consisting of a variable region (VL) of SEQ ID NO:2, wherein aa 96 denotes proline or isoleucine, aa 100 denotes proline or glutamine, aa 103 denotes arginine or lysine, aa 104 denotes valine or leucine and aa 105 denotes aspartic acid or glutamic acid, and of a human light chain constant region (CL).
in a prokaryotic or eukaryotic host cell and recovering said antibody from said cell. The constant regions provide C1q complement binding and are therefore preferably of human IgG1 type. Preferably, the heavy chain variable region contains the amino acid combination of

aa 30 Arg, aa 31 Asn, aa 94 Tyr and aa 104 Asp or
aa 30 Arg, aa 31 Ser, aa 94 Tyr and aa 104 Asp or
aa 30 Ser, aa 31 Asn, aa 94 His and aa 104 Glu.

Preferably, the light chain variable region contains the amino acid combination of

aa 96 Pro, aa 100 Pro, aa 103 Lys, aa 104 Val and aa 105 Asp (antibody 1A and 8),
aa 96 Ile, aa 100 Gln, aa 103 Arg, aa 104 Leu and aa 105 Glu (antibody 23).

The combination aa 30 Arg, aa 31 Asn, aa 94 Tyr and aa 104 Asp in the heavy chain and aa 96 Pro, aa 100 Pro, aa 103 Lys, aa 104 Val and aa 105 Asp in the light chain is especially preferred.

The invention further comprises the use of an antibody according to the invention for the diagnosis of IGF-IR in vitro, preferably by an immunological assay determining the binding between IGF-IR of a sample and the antibody according to the invention.

In another aspect, the present invention provides a composition, e.g. a pharmaceutical composition, containing one or a combination of human monoclonal antibodies, or the antigen-binding portion thereof, of the present invention, formulated together with a pharmaceutically acceptable carrier.

Pharmaceutical compositions of the invention also can be administered in combination therapy, i.e., combined with other agents. For example, the combination therapy can include a composition of the present invention with at least one anti-tumor agent or other conventional therapy.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Taxotere (docetaxel), Busulfan, Cytoxin, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincreistine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins, Esperamicins (see US Patent No. 4,675,187), Melphalan and other related nitrogen mustards.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes, chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial fungal, plant or animal origin, or fragments thereof.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" Biochemical Society Transactions, 14, pp. 375-382, 615th Meeting Belfast (1986), and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," Directed Drug Delivery, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate- containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam ring prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (e.g. by injection or infusion).

A "pharmaceutically acceptable salt" refers to a salt that retains the desired biological activity of the antibody and does not impart any undesired toxicological effects (see e.g. Berge, S.M., et al., J. Pharm. Sci. 66 (1977) 1-19). Such salts are included in the invention. Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric salts.

A composition of the present invention can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results.

To administer a compound of the invention by certain routes of administration, it may be necessary to coat the compound with, or co- administer the compound

with, a material to prevent its inactivation. For example, the compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions.

Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, supra, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention may be varied so as to obtain an amount of the active

ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

The composition must be sterile and fluid to the extent that the composition is deliverable by syringe. In addition to water, the carrier can be an isotonic buffered saline solution, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof.

Proper fluidity can be maintained, for example, by use of coating such as lecithin, by maintenance of required particle size in the case of dispersion and by use of surfactants. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol or sorbitol, and sodium chloride in the composition. Long-term absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate or gelatin.

When the active compound is suitably protected, as described above, the compound may be orally administered, for example, with an inert diluent or an assimilable edible carrier.

The antibodies according to the invention can be used for the treatment of a patient suffering from a tumor disease and in the need of an antitumor therapy. Therefore, the invention comprises a method for the treatment of a tumor patient, preferably a patient suffering from cancer, especially from colon, breast, prostate and lung cancer.

The invention further provides a method for the manufacture of a pharmaceutical composition comprising an effective amount of an antibody according to the

invention together with a pharmaceutically acceptable carrier and the use of the antibody according to the invention for such a method.

The invention further provides the method of treatment, method of manfacture and the pharmaceutical composition of an antibody according to the invention together with a chemotherapeutic, preferably cytotoxic agent or a prodrug thereof.

In addition, the antibodies can be used in combination with a cytotoxic radiotherapy.

The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figure 1 IGF-IR surface expression in low and high density cell culture.

Figure 2 WST assay for proliferation inhibition in 3D culture.

Figure 3 Primary tumor volume measured during treatment until day 55; Vehicle: 1; antibody 1A 20 mg/kg: 2; antibody 1A 7 mg/kg: 3; antibody 1A 2 mg/kg: 4.

Figure 4 Inhibition of I^{125}-IGF-I binding to HT29 cells by antibodies 1A, 8 and 23.

Figure 5 Inhibition of I^{125}-IGF-I binding to various human tumor cell lines by antibodies against hIGF-1R.

Figure 6 Inhibition of I^{125}-IGF-II binding to HT29 cells by antibody 1A.

Figure 7 Inhibition of I^{125}-IGF-II bindingto HT29 cells by antibody αIR3.

Figure 8 Blockage of IGF-I induced phosphorylation of both IGF-IR and AKT/PkB.

Figure 9 Downregulation of IGF-IR protein level on tumor cells.

Figure 10 No inhibition of I^{125}-insulin binding to 3T3-IR cells by anti-hIGF-1R antibodies. (MAX w/o Ab: maximal binding of I^{125}-insulin; MIN: minimal binding after competition with 1 µM insulin)

Figure 11 Induction of downregulation of IGF-IR in vivo.

Figure 12 Crossreactivity of antibodies with IGF-IR from other species.

Example 1

Generation of a hybridoma cell line producing anti-IGF-IR antibodies

Culture of hybridomas

Generated HuMab hybridomas were cultured in Hybridoma Express Medium (PAA Laboratories GmbH, Austria) supplemented with 2 mM L-glutamine (BioWhittaker) and 4% Origen Cloning Factor (Igen, France) at 37°C and 5% CO_2; or in Iscoves Modified Dulbeco's Medium (500 ml: BioWhittaker Europe, Belgium) supplemented with Fetal Clone Serum (50 ml: Hyclone, Utah), and Origen Hybridoma Cloning Factor (30 ml: Igen, Gaithersburg MD) at 37°C and 5% CO_2.

Immunization procedure of transgenic mice

Ten HCo7 transgenic mice (4 males and 6 females), strain GG2201 (Medarex, San José, CA, USA) were alternatingly immunized with $1x10^6$ NIH 3T3 cells, transfected with an expression vector for IGF-IR, and 20 µg soluble extracellular domain of IGF-IR. Six immunizations were performed in total, three intraperitoneal (IP) immunizations with the IGF-IR expressing cells and three subcutaneous (SC) immunizations at the tail base with the recombinant protein. For the first immunization, 100 µl of $1x10^6$ NIH 3T3 IGF-IR cells was mixed with 100 µl complete Freunds' adjuvant (CFA; Difco Laboratories, Detroit, USA). For all other

immunizations, 100 µl of cells in PBS were used or recombinant protein was mixed with 100 µl incomplete Freunds' adjuvant (ICFA; Difco).

Antigen specific ELISA

Anti-IGF-IR titers in sera of immunized mice were determined by antigen specific ELISA. IGF-IR soluble extracellular domain at a concentration of 1 µg/ml in PBS was coated overnight at 4°C, or for two hours at 37°C, to 96 wells plates. Thereafter, the wells were blocked with PBSTC (PBS supplemented with 0.05% Tween®-20 and 2% chicken serum (Gibco BRL)) for 1 hour (h) at room temperature. First tap sera were diluted 1/50 in PBSTC, sera from all other taps were pre-diluted 1/100 in PBSTC and serially diluted up to 1/6400. Diluted sera were added to the wells and incubated for 1 h at 37°C. Pre-tap serum was used as negative control. 200 ng/ml goat anti-human IGF-IR (100 µg/ml) was used as positive control. Subsequently, plates were washed twice with PBST and incubated with horse radish peroxidase (HRP)-conjugated rat anti-human IgG $F(ab')_2$ (DAKO), diluted 1/2000 in PBSTC for 1 h at 37°C. Wells were washed twice with PBST and assays were developed with freshly prepared ABTS® solution (1 mg/ml) (ABTS: 2,2'-azino bis (3-ethylbenzthiazoline-6-sulfonic acid) for 30 minutes at room temperature (RT) in the dark. Absorbance was measured at 405 nm.

FACS analysis

In addition to determination by antigen specific ELISA, anti-IGF-IR titers in sera of immunized mice were also determined by FACS analyses. NIH 3T3 IGF-IR cells and the parental NIH 3T3 cells were incubated with diluted sera for 30 minutes at 4°C. Alternating IP and SC immunizations were performed at two weeks intervals starting with an IP immunization. Pre-tap serum (parental NIH 3T3 cells) was used as negative control. Initially, 200 ng/ml goat anti-human IGF-IR was used as positive control. Cells were washed three times in PBS supplemented with 1% bovine serum albumin and 0.01% azide. Subsequently, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated antigen binding fragments ($F(ab')_2$ fragments) of rat anti-human human IgG diluted 1/100 in FACS buffer, for 30 minutes at 4°C. Cells were washed twice in FACS buffer and samples were analyzed on a FACSCalibur (Becton Dickinson, Erembodegem-Aalst, Belgium).

Boosting of mice

When serum titers of anti-IGF-IR were found to be sufficient, mice were additionally boosted twice with 15 µg IGF-IR extracellular domain in 200 µl PBS intravenously (i.v.) 4 and 3 days before fusion.

Hybridoma generation

Mice were sacrificed and the spleen and lymph nodes flanking the abdominal aorta and vena cava were collected. Fusion of splenocytes and lymph node cells with the fusion partner SP 2.0 cells was performed according to standard operating procedures.

κ-ELISA

To determine whether hybridomas that resulted from the fusion generate human antibodies, a κ-ELISA was performed. ELISA plates were coated with rat anti-human IgG κ-light chain antibody (DAKO) diluted 1/10000 in PBS by overnight incubation at 4°C. After discarding the wells, plates were blocked by incubation with PBSTC for 1 hour at room temperature. Thereafter, wells were incubated with hybridoma culture supernatant, 1/2 diluted in PBSTC. Culture medium 1/2 diluted in PBSTC was used as negative control, κ-light positive mouse serum 1/100 diluted in PBSTC served as positive control. Subsequently, wells were washed thrice and were incubated with HRP-conjugated rat anti-human IgG $F(ab')_2$ (DAKO), diluted 1/2000 in PBSTC for 1 h at 37°C. Wells were washed thrice and assays were developed with freshly prepared ABTS® solution (1 mg/ml) for 30 minutes at room temperature (RT) in the dark. Absorbance was measured at 405 nm in an ELISA plate reader.

Three monoclonal antibodies were prepared.

Antibody 1A: SEQ ID NOS:1 and 2
Antibody 8: SEQ ID NOS: 3 and 4
Antibody 23: SEQ ID NOS: 5 and 6.

Example 2

Determination of the affinity of anti-IGF-IR antibodies to IGF-IR

Instrument: BIACORE® 3000
Chip: CM5
Coupling: amine coupling
Buffer: HBS (HEPES, NaCl), pH 7.4, 25°C

For affinity measurements anti human Fcγ antibodies (from rabbit) have been coupled to the chip surface for presentation of the antibody against IGF-IR. IGF-IR extracellular domain was added in various concentrations in solution. Association was measured by an IGF-IR-injection of 3 minutes; dissociation was measured by washing the chip surface with buffer for 5 minutes. The affinity data for antibodies 1A, 8 and 23 are shown in Table 1.

Table 1:
Affinity data measured by SPR (BIACORE® 3000)

Antibody	ka (1/Ms)	kd (1/s)	KA (1/M)	KD (M)
1A	1.18×10^5	4.68×10^{-5}	2.52×10^9	3.97×10^{-10}
8	8.18×10^4	1.61×10^{-4}	4.98×10^8	2.01×10^{-9}
23	8.41×10^4	1.63×10^{-5}	5.17×10^9	1.94×10^{-10}

Example 3

WST proliferation assay

To assess the capacity of HuMab antibodies to inhibit IGF-I-induced proliferation of IGF-IR expressing cell lines, the WST-1 proliferation assay was performed. The IGF-IR expressing cell line NIH 3T3 was cultured for two days in starvation medium (i.e., regular culture medium with 0.5% FCS instead of 10% FCS), $9x10^3$ cells per well in 96 wells tissue culture plates in order to bring back the metabolic system to a base level. Thereafter, medium was removed and wells were replenished with 100 µl starvation medium containing the following compounds: 1) 10^{-9} M IGF-I; 2) 10^{-9} M IGF-I plus 10 µg/ml protein-A purified HuMab antibody; 3) 10^{-9} M IGF-I plus 10 µg/ml αIR3. As negative controls, cells were incubated with starvation medium, as positive control cells were incubated with culture medium

only. Cells were cultured for an additional two days. Subsequently, 10 µl of WST-1 reagent (Roche Diagnostics GmbH) was added to the wells to detect live cells. After 2-3 hours, absorbance was measured at 450 nm in an ELISA plate reader. The percentage of inhibition of proliferation was calculated according to the following formula:

$OD_{100\%\ growth} = OD_{IGF\text{-}I} - OD_{starvation\ medium}$

Inhibition of proliferation: $(1 - OD_{sample} / OD_{100\%\ growth}) \times 100\%$

Example 4

Three-dimensional growth of tumor cells and overexpression of IGF-I receptor at cell-cell-contact (3D culture)

Materials and Methods:

NCI H322M cells were cultured in RPMI media on optical grade glass cover slides either at low density or superconfluent to study the effects on IGF-IR surface expression. In parallel, H322M xenograft tissue isolated from the control group (untreated mice) was shock frozen in isopentane and kryosections were cut at 5µm thickness. Immunofluorescence labelling was performed using a mouse-anti IGF-IR monoclonal antibody (αIR3, 5µg/ml) or an antibody according to the invention, followed by a goat anti-mouse-antibody or a goat anti-mouse antibody labeled with Cy3. Specimens were imaged on a Leica SP2 confocal microscope or analyzed by FACS.

Results:

When H322M cells cultured at high density were imaged by confocal microscopy it became apparent that IGF-IR clustered specifically at the sites of cell-cell contact. When compared to H322M cells grown in vivo, i.e. xenograft tissue, there was a striking similarity to densely packed in vitro cultures as far as the organization of surface IGF-I receptors was concerned.

The upregulation of surface IGF-I receptor in superconfluent cultures of H322M cells was also quantified by FACS. IGF-I receptor surface expression increased more than 10 fold when cells were grown under high density conditions compared to low density without significant cell-cell contacts.

Other tumor cells such as HT29, MDA231 and MCF-7 showed a similar behavior, indicating that upregulation of IGF-I receptors on the cell surface upon establishing cell-cell contact sites is not an unique feature of H322M cells but appears to be a general property of a more tissue like organization that is also found in vivo (Fig. 1).

Growth inhibition of H322M tumor cells expressing IGF-IR in 3D culture under treatment with antibody 1A

Materials and Methods:

H322M cells were cultured in RPMI1640/10% NCS media on poly-HEMA (poly(2-hydroxyethylmethacrylate)) coated dishes to prevent adherence to the plastic surface. Under these conditions H322M cells form dense spheroids that grow three dimensionally (a property that is called anchorage independence). These spheroids represent the three dimensional histoarchitecture and organization of solid tumors in situ. Spheroid cultures were incubated for 9 days in the presence of increasing amounts of antibodies from 0-10µg/ml. Two nonspecific antibodies (antibody against HBV and against E25) were used as a negative control. The WST conversion assay was used to measure growth inhibition.

Results:

When H322M spheroid cultures were treated with different concentrations of antibody 1A (0.32-10µg/ml) a dose dependent inhibition in growth could be observed, while the control antibodies against HBV and E25 (anti-IgE) had little or no effect. The reduction in WST for antibody 1A is therefore primarily due to a reduced proliferation of the cells (Fig. 2).

Example 5

Determination of pharmacokinetic properties

Antibody 1A was administered in two different pharmacokinetic studies. For the first study, the antibody was administered formulated as a potassium phosphate buffer solution. For the second study, the antibody was administered formulated as a sodium chloride/ histidine solution.

5a) **First animal study**

Female NMRI mice were used (18 - 23 g body weight). Antibody 1A was given as a solution (KPO4) for i.v., and i.p. administration route.

Doses:

10 mg/kg i.v. Drug concentration: 1mg/mL. Administered volume: 10 mL/kg.
10 mg/kg i.p. Drug concentration: 1mg/mL. Administered volume: 10 mL/kg.
Single dose administration.

Plasma samples were subsequently analysed for plasma levels of the compound using a human IgG-ELISA method.

Reagents:

Antibodies: Capture antibody: polyclonal rabbit antibody against human kappa-light chains IgG (Dako, Code No. A0191)

Detection antibody: polyclonal rabbit antibody against human IgG, conjugate with Horse raddish peroxidase (DAKO, Code No. P0214)

Assay procedure:

Coating of the microtiter plate:

Step

- dilute capture antibody 1:10000 with 100mM Na-Carbonate, pH=9,6
- add 100 µl of this solution (Step 1) to each well
- incubate the plate at 4°C over night (12h)
- remove solvents of each well
- wash 3 times with 300 µl/well PBST

Adding animal sample and calibration sample, respectively dilute animal sample (range 1 to 10 up to 1 to 200000) and calibration sample, respectively (concentration 0.625; 1.25; 2.5; 5; 10; 20 and 40 ng/ml human IgG) with 3% BSA/PBST:

- add 100µl animal/calibration sample to each well
- incubate for 1 h at room temperature (22°C)

- wash 3 times with 300 µl/well PBST

Detection:

- dilute detection antibody 1:2000 with 3% BSA/PBST
- add 100 µl to each well
- incubate for 1 h at room temperature (22 °C)
- wash 3 times with 300 µl/well PBST
- add 100µl ABTS®-solution to each well
- after ca. 10 min stop the color reaction with 50 µl/well 0.5 M oxalic acid
- measure the extinction at 405nm

PK Parameters:

Table 2:
Pharmacokinetic parameters of antibody 1A fomulated as a potassium phosphate buffer

Parameter	units		
Species		Mouse	Mouse
Strain		NMRI	NMRI
Gender		female	female
Formulation		Phosphate buffer	Phosphate buffer
Dose	mg/kg	10.0	10.0
Administration route		i.v.	i.p.
CMAX	ng/mL	208000	68800
CMAX_NORM	ng/mL / mg/kg	20800	6880
TMAX	h	0	96
AUC_0_LST	h.ng/mL	36300000	32300000
AUC_0_LST_NORM	h.ng/mL / mg/kg	3630000	3230000
AUC_0_INF	h.ng/mL	40200000	36300000
AUC_0_INF_NORM	h.ng/mL / mg/kg	4020000	3630000
PCT_AUC_EXTRA	%	9.75	11.00
MRT_LST	h	324	348
MRT_INF	h	437	475
CL_TOTAL	mL/min/kg	0.00414	
VZ	L/kg	0.11	
VSS	L/kg	0.1	
HALFLIFE_Z	h	308 (=12.8 days)	324 (=13.5 days)
F	%	100.0	89.0

5

5b) Second animal study

Animals:

Female NMRI mice were used (21 - 28 g body weight). Antibody 1A was given as a solution (histidine/sodium chloride) for i.v., and i.p. administration route.

Doses:

10 mg/kg i.v. Drug concentration:	1 mg/mL.	Administered volume:	10 mL/kg.
2 mg/kg i.p. Drug concentration:	0.2 mg/mL.	Administered volume:	10 mL/kg.
20 mg/kg i.p. Drug concentration:	2 mg/mL.	Administered volume:	10 mL/kg.

Single dose administration.

Plasma samples were subsequently analysed for plasma levels of the compound using a human IgG-ELISA method. Calibration was done using the antibody 1A for preparing calibration samples.

Reagents and assay procedure: see Example 5a

PK Parameters:

Table 3:

Pharmacokinetic parameters of antibody 1A fomulated as a histidine/sodium chloride solution

Species		Mouse	Mouse	Mouse
Strain		NMRI	NMRI	NMRI
Gender		female	female	female
Formulation		solution (NaCl/histidine)	solution (NaCl/histidine)	solution (NaCl/histidine)
Dose	mg/kg	10	2	20
Administration route		i.v.	i.p.	i.p.
CMAX	ng/mL	222000	27900	186000
CMAX_NORM	ng/mL / mg/kg	22200	14000	9300
TMAX	h	0	24	24
AUC_0_LST	h.ng/mL	30500000	4920000	59000000
AUC_0_LST_NORM	h.ng/mL / mg/kg	3050000	2460000	2950000
AUC_0_INF	h.ng/mL	36700000	6910000	72100000
AUC_0_INF_NORM	h.ng/mL / mg/kg	3670000	3460000	3610000
PCT_AUC_EXTRA	%	16.90	28.80	18.20
MRT_LST	h	247	217	239
MRT_INF	h	381	513	389
CL_TOTAL	mL/min/kg	0.00454		
CL_TOTAL_CTG	L,M,H			
CL_ORAL	mL/min/kg		0.00482	0.00462
VZ	L/kg	0.102		
VZ_ORAL	(Vz/F) L/kg		0.166	0.108
VSS	L/kg	0.1		
VSS_CTG	L,M,H	L		
HALFLIFE_Z	h	260.0	399.0	270.0
F	%	100.0	80.7	96.7

Abbreviations:

Abbreviation	Meaning
CMAX	Cmax
CMAX_NORM	Cmax dose-normalized
TMAX	Tmax
AUC_0_INF	AUC extrapolated
AUC_0_LST	AUC observed
AUC_0_INF_NORM	AUC extrapolated, normalized
AUC_0_LST_NORM	AUC observed, normalized
PCT_AUC_EXTRA	percentage AUC extrapolated
CL_TOTAL	Total Clearance
VSS	Steady state distribution volume
VZ	Terminal distribution volume
MRT_INF	Mean residence time (extrapolated)
MRT_LST	Mean residence time (observed)
HALFLIFE_Z	Terminal half-life
F	Bioavailability (i.v. $\hat{=}$ 100%)

Example 6

Pharmacodynamic testing of recombinant anti-IGF-IR antibody 1A

The effects of the antibody were investigated in vivo. Tumors were induced in athymic nude mice according to established methods. Human H322M cells were coinjected together with Matrigel subcutaneously into 6-7 week-old athymic nude mice (nu/nu). For that purpose, 5 x 10^6 H322M cells were concentrated in 100 µl culture medium and mixed with 100 µl Matrigel®. 200 µl of this mixture were injected into the right flanks of the mice. Treatment was initiated when induced tumors reached an average volume of 125 mg. Tumor volume was calculated by measuring tumor diameters with Vernier calipers twice a week according to the formula

$$\text{tumor volume [mg]} = (\text{length} \times (\text{width})^2$$

(Gallicchio, M.A., et al., Int. J. Cancer 94 (2001) 645-651).

All antibodies were administered intraperitoneally (i.p.) at 10 ml/kg.

After tumors had grown to an average volume of 100 mg the antibody was administered twice a week i.p. at 20 mg/kg, 7 mg/kg and 2 mg/kg twelve times starting treatment with a doubled loading dose given once on the first day of the treatment period. All three doses of the antibody had an effect on primary tumor volume. Figure 3 shows the tumor size in relation to the various treatments over time. The experiment demonstrates that blocking of the IGF-IR axis by antibody 1A results in good antitumor effects.

Example 7

Inhibition of IGF-I and IGF-II binding to tumor cells expressing IGF-IR

In order to determine the ability of the antibody of the invention to block binding of the ligands IGF-I and IGF-II to the IGF-I receptor (IGF-IR), competition experiments with radioactively labeled ligand peptides were performed.

Human tumor cells (HT29, NCI H322M, 0.5 to 1 x 10^5/ml) were plated in RPMI 1640 medium (PAA, Cat. No. E15-039) supplemented with 2 mM L-Glutamin, 1x non-essential amino acids (Gibco, Cat. No. 11140-035), 1 mM sodium pyruvate (Gibco, Cat. No. 11360-039) and 10% heat inactivated FCS (PAA, Cat. No. A15-771). Six bottles in the T175 format were inoculated with 20 ml cells in the respective medium for each experiment and cultivated for two days at 37°C and 5% CO_2 to obtain confluent cell monolayers.

To collect individual cells, 2 ml of 1x Trypsin/EDTA (Gibco, Cat. No. 25300-054) per T175 flask were added and detachment of cells monitored with a Zeiss Axiovert25 microscope. The cells were collected and medium with 10% FCS as described before was added to a total volume of 50 ml. Cells were reisolated by centrifugation for 10 minutes at 1000 rpm (Heraeus sepatech, Omnifuge 2.0 RS) and resuspended in 50 ml of binding buffer (120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1 mM EDTA, 10 mM D(+)glucose, 15 mM NaAc, 100 mM Hepes pH 7.6, 1% BSA). Cells were counted, reisolated by centrifugation and adjusted with binding buffer to 1 x 10^6 cells/ml.

I^{125}-labeled IGF-I and IGF-II peptides (Amersham, ~2000 Ci/mmol, Cat. No. IM172 and IM238), solubilized in 0.1% CH_3COOH, were diluted in binding buffer to a final activity of 4×10^5 counts/(minute x ml). 75 µl of antibody at the specified concentrations together with 25 µl of prediluted I^{125}-labeled IGF-I or IGF-II peptide was added to 200 µl of cell suspension and incubated for 3,5 h at 4°C. Cells were reisolated by centrifugation for 5 minutes at 2000 rpm (Eppendorf, 5415C) and supernatant removed. After washing two times in 1 ml binding buffer, cells were resuspended in 1 ml binding buffer and transferred to scintillation tubes. The amount of radioactive peptide bound to the cell surface receptors was measured on a scintillation counter.

The resulting IC_{50} curves demonstrating the ability of the antibody to inhibit binding of IGF-I and IGF-II peptide to the IGF-I receptor are shown in Figs. 4, 5 and 6. The results for antibody αIR3 are shown in Figs. 5 and 7.

Example 8

Antibody competition assay for IGF-IR binding

For an epitope mapping of anti-IGF-IR monoclonal antibodies a similar format as for affinity measurement (Example 2) was selected. Antibody 1a was bound to anti human Fcγ antibodies (from rabbit) which were amine-coupled to the chip surface. To determine whether another antibody was directed against an IGF-IR epitope overlapping with the epitope recognized by antibody 1a, IGF-IR was pre-incubated with this antibody under saturating conditions in solution. After incubation for at least 30 minutes at RT the IGF-IR with pre-bound antibody was injected to the flow cell and binding to the reference antibody 1a at the chip surface was monitored. In case of overlapping epitope recognition of test antibody and reference antibody 1A binding of IGF-IR was inhibited by at least 10% compared to the binding signal of IGF-IR alone (100% binding signal) at a standard concentration of 50 nM. In case of additional antibody binding the IGF-IR binding signal was increased by at least 10% indicating the recognition of an independent binding epitope on IGF-IR by the test antibody.

Example 9

Inhibition of IGF-I mediated phosphorylation of IGF-IR and Akt/PKB

In order to determine the ability of the antibody of the invention to inhibit activation and phosphorylation of the IGF-I receptor (IGF-IR), competition experiments were performed with IGF-I peptide and subsequent Western blotting analysis with antibodies specific for phosphorylated tyrosine.

Human tumor cells (HT29, NCI H322M, 5 x 10^4/ml) were plated in RPMI 1640 medium (PAA, Cat. No. E15-039) supplemented with 2 mM L-Glutamin, 1x non-essential aminoacids (Gibco, Cat. No. 11140-035), 1 mM sodium pyruvate (Gibco, Cat. No. 11360-039) and 0.5% heat inactivated FCS (PAA, Cat. No. A15-771). For determination of IC_{50} values, 12 well plates were inoculated with 1 ml cells in the respective medium for each experiment and cultivated for two days at 37°C and 5% CO_2.

After 48 hours of cultivation with low serum medium, the medium was carefully removed and replaced by different concentrations of antibody diluted in the respective medium. After 5 minutes incubation at 37°C and 5% CO_2 IGF-I peptide was added at a final concentration of 2 nM and cells were again incubated for 10 minutes under the conditions mentioned above. The medium was carefully removed by aspiration and 100 µl of cold lysis buffer was added per well (50mM Hepes pH 7.2, 150 mM NaCl, 1mM EGTA, 10% glycerol, 1% Triton®-X100, 100mM NaF, 10 mM $Na_4P_2O_7$, Complete® protease inhibitor). The cells were detached using a cell scraper (Corning, Cat. No. 3010) and well contents transferred to Eppendorf reaction tubes. Cell fragments were removed by centrifugation for 10 minutes at 13000 rpm and 4°C and half of the supernatant was added to 2x Laemmli sample buffer in a 1:1 (v/v) ratio. For immunoprecipitation of IGF-IR, the remaining supernatant of cell lysates underwent a clearifying spin (10 minutes at 13000 rpm and 4°C) right before 1 µl of primary antibody was added (C-20, Santa Cruz Biotechnologies, mAb 24-55, GroPep). After 2 hours incubation at 4°C in a rotating Eppendorf reaction tube, 25 µl Protein G Sepharose® beads (Amersham Biosciences, Cat. No. 17-0618-01) were added followed by another incubation step of 1 hour at 4°C. The beads with bound antibody-protein-complexes were isolated by centrifugation (1 minute at 2000 rpm and 4°C) and washed three times with wash buffer (lysis buffer with only 0.1% Triton®-X100).

After boiling the beads in Laemmli sample buffer, cellular proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (PROTRAN® BA 85, Schleicher&Schuell) by semi-dry Western blotting.

A phosphotyrosin specific antibody (<P-Tyr>, Upstate, clone 4G10, Cat. No. 05-321) was used to determine phosphorylation status of immunopurified IGF-IR. For the detection of phosphorylated Akt/PKB an antibody with specificity for phosphorylated Ser473 (<P-PkB>, Cell Signalling, Cat. No. 9271) was applied.

The observed blockage of IGF-I induced phosphorylation of both IGF-IR and Akt/PKB is shown in Fig. 8.

Example 10

Induction of antibody mediated downregulation of IGF-IR in-vitro

In order to detect effects of the antibody of the invention on the amount of IGF-I receptor (IGF-IR) in tumor cells, time-course experiments and subsequent western-blotting analysis with IGF-IR specific antibodies were performed.

Human tumor cells (H460, QG56, MCF-7, 5 x 10^4 cells/ml) were plated in RPMI 1640 medium (PAA, Cat. No. E15-039) supplemented with 2 mM L-Glutamin, 1x non-essential aminoacids (Gibco, Cat. No. 11140-035), 1 mM sodium pyruvate (Gibco, Cat. No. 11360-039) and 10% heat inactivated FCS (PAA, Cat. No. A15-771). For each incubation period one 12 well plate was inoculated with 1 ml cells in the respective medium for each experiment and cultivated for 24 hours at 37°C and 5% CO_2.

The medium was carefully removed and replaced by different concentrations of antibody diluted in the respective medium. In two control wells, medium was replaced by either medium without antibody or medium with a control antibody (AB-1, Oncogene, Cat. No. GR11). Cells were incubated at 37°C and 5% CO_2 and individual plates were taken out for further processing after 15 minutes, 24 hours and 48 hours.

The medium was carefully removed by aspiration and 100 µl of cold lysis buffer was added per well (50mM Hepes pH 7.2, 150 mM NaCl, 1mM EGTA, 10% glycerol,

1% Triton®-X100, 100mM NaF, 10 mM $Na_4P_2O_7$, Complete® protease inhibitor). The cells were detached using a cell scraper (Corning, Cat. No. 3010) and well contents transferred to Eppendorf reaction tubes. Cell fragments were removed by centrifugation for 10 minutes at 13000 rpm and 4°C and the supernatant was added to 2x Laemmli sample buffer in a 1:1 (v/v) ratio. Cellular proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (PROTRAN® BA 85, Schleicher&Schuell, Cat. No. 10 401196) by semi-dry western-blotting.

An antibody specific for IGF-IR (<IGF-IR>, C-20, Santa Cruz Biotechnologies, Cat. No. sc-713) was used to determine protein levels of IGF-IR.

Downregulation of IGF-IR induced by the antibody of the invention after less than 24 hours after addition of the antibody was observed. The results are shown in Fig. 9.

Example 11

Inhibition of insulin binding to 3T3-cells expressing human insulin receptor

In order to determine whether the antibody of the invention also blocks binding of insulin to the insulin receptor (IR), competition experiments were performed with a radioactively labeled ligand peptide.

NIH-3T3 cells (1 x 10^5/ml) expressing recombinantly high numbers (>10^5) human IR were plated in MEM Dulbecco medium (DMEM) with high glucose (PAA, Cat. No. E15-009) supplemented with 2mM L-Glutamin (Gibco, Cat. No. 25030-024) and 10% heat inactivated FCS (PAA, Cat. No. A15-771). Six bottles in the T175 format were inoculated with 20 ml cells in the respective medium for each experiment and cultivated for two days at 37°C and 5% CO_2 to obtain confluent cell monolayers.

To collect individual cells, 2 ml of 1x Trypsin/EDTA (Gibco, Cat. No. 25300-054) per T175 flask were added and detachment of cells monitored with a microscope. The cells were collected and medium with 10% FCS as described before was added to a total volume of 50 ml. Cells were reisolated by centrifugation for 10 minutes at 1000 rpm and resuspended in 50 ml of binding buffer (120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1 mM EDTA, 10 mM D(+)glucose, 15 mM NaAc, 100 mM Hepes

pH 7.6, 1% BSA). Cells were counted, reisolated by centrifugation and adjusted with binding buffer to 1 x 10^6 cells/ml.

I^{125}-labeled insulin peptide (Amersham, Cat. No. IM166, ~2000 Ci/mmol), solubilized in 0.1% CH_3COOH, were diluted in binding buffer to a final activity of $4x10^5$ counts/(minute x ml). 75 µl of antibody together with 25 µl of prediluted I^{125}-labeled insulin peptide was added to 200 µl of cell suspension (final antibody concentration 200 nM) and incubated for 3,5 h at 4°C. Cells were reisolated by centrifugation for 5 minutes at 2000 rpm and supernatant was removed. After washing two times in 1 ml binding buffer, cells were resuspended in 1 ml binding buffer and transferred to scintillation tubes. The amount of radioactive peptide bound to the cell surface receptors was measured on a scintillation counter.

The results demonstrate that the antibody of the invention does not interfere with binding of insulin ligand to the insulin receptor (Fig. 10).

Example 12

Induction of receptor down-regulation in different tumor types

Human tumors (NCI H322M or NCI H460) were induced in nude mice and treated with the antibody of the invention as described in Example 6. After termination of the experiment, the tumors were extracted and homogenized under liquid nitrogen. Cold lysis buffer was added (50mM Hepes pH 7.2, 150 mM NaCl, 1mM EGTA, 10% glycerol, 1% Triton-X100, 100mM NaF, 1 mM Na_3V0_4, 10 mM $Na_4P_2O_7$, Complete® protease inhibitor, 1mM PMSF) in a buffer-volume to tumor-weight ratio of 3:1 and thoroughly mixed with the thawing tumor homogenate. After solubilizing the tissue for 15 minutes on ice, insoluble fragments were removed by centrifugation for 10 minutes at 13000 rpm and 4°C. The protein concentration of the samples was determined with the Micro BCA® Reagents (Pierce) and lysis buffer was added to adjust equal concentrations. Part of the supernatant was added to 2x Laemmli sample buffer in a 1:1 (v/v) ratio. Cellular proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (PROTRAN® BA 85, Schleicher&Schuell, Cat. No. 10 401196) by semi-dry western-blotting. An IGF-IR specific antibody (C-20, Santa Cruz Biotechnologies, Cat. No. sc-713) was used to detect IGF-IR.

A dramatic decrease of IGF-IR levels in all tumors treated with the antibody of the invention (Fig. 11) corresponding to the results of the in vitro downregulation experiments was observed.

Example 13

Antibody crossreactivity with IGF-IR from rat, mouse, Marmoset and Cynomolgus

The antibody of the invention was tested for its ability to bind to the IGF-I receptor of other species. Immunoprecipitation experiments were performed with the antibody of the invention and tissue or cell lysates from different animal species.

Frozen animal tissue was homogenized under liquid nitrogen. Cold lysis buffer was added (50mM Hepes pH 7.2, 150 mM NaCl, 1mM EGTA, 10% glycerol, 1% Triton-X100, 100mM NaF, 1 mM Na_3V0_4, 10 mM $Na_4P_2O_7$, Complete® protease inhibitor, 1mM PMSF) in a buffer-volume to tissue-weight ratio of 3:1 and thoroughly mixed with the thawing tissue homogenate. After solubilizing the tissue for 15 minutes on ice, insoluble fragments were removed by centrifugation for 10 minutes at 13000 rpm and 4°C. The protein concentration of the samples was determined with the Micro BCA® Reagents (Pierce) and lysis buffer was added to adjust equal concentrations. Animal cells were solubilized according to the protocol for tumor cell lines (Example 10).

For immunoprecipitation of IGF-IR, cell and tissue lysates (1 mg total protein) underwent a clarifying spin (10 minutes at 13000 rpm and 4°C) right before 2 µg of primary antibody 1A was added. The same experiment was also performed with an unrelated human control antibody directed against hepatitis B virus protein (HBV) as a measure for unspecific binding. After 2 hours incubation at 4°C in a rotating eppendorf reaction tube, 25 µl Protein G Sepharose® beads (Amersham Biosciences, Cat. No. 17-0618-01) were added followed by another incubation step of 1 hour at 4°C. The beads with bound antibody-protein-complexes were isolated by centrifugation (1 minute at 2000 rpm and 4°C) and washed three times with wash buffer (lysis buffer with only 0.1% Triton®-X100). After boiling the beads in Laemmli sample buffer, cellular proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (PROTRAN® BA 85, Schleicher&Schuell, Cat. No. 10 401196) by semi-dry western-blotting.

An IGF-IR specific antibody with broad species cross-reactivity (C-20, Santa Cruz Biotechnologies, Cat. No. sc-713) was used to determine the levels of IGF-IR immunoprecipitated with the antibody of the invention.

Cross-reactivity with IGF-IR from Cynomolgus and Marmoset monkey (Fig. 12), but no cross-reactivity to the rat or mouse IGF-I receptor was observed.

Example 14

C1q Binding ELISA

Introduction

To determine the ability of antibodies according to the invention to fix C1q an ELISA approach was used. C1q is part of the adaptive immune system and, upon binding to immune complexes, triggers the sequential activation of several zymogens. The enzymes in turn, cause the cleavage of C3 molecules, which can result in the onset of inflammatory reactions, opsonization of foreign or aberrant particles and lysis of cell membranes.

In principle, the ELISA plate is coated with concentration ranges of the antibody, to which human C1q is added. C1q binding is detected by an antibody directed against human C1q followed by a peroxidase-labeled conjugate.

Materials and methods

Antibody 1A, 8 and 23 and control antibodies were tested in concentrations of 10, 5, 1 and 0.5 µg/ml. Table 1 shows the specificities of the samples tested. As a negative control a human IgG4 (CLB, stock 0.5 µg/ml), that binds C1q very weakly, was used. Human IgG1 was incorporated as positive control. Human C1q stock solution with a concentration of 2 µg/ml was used. For the detection of C1q a rabbit antibody directed against C1q (Dako) and an anti-rabbit IgG antibody, conjugated with horseradish peroxidase (Sigma) were used.

Calculations and curve fitting

Calculations concerning maximum binding (Bmax) of the HuMab tested were determined using nonlinear regression curve fitting (one site binding) using Graphpad Prism software.

Results

The antibodies according to the invention show dose dependent binding of human C1q protein. The optical density at 405 nm (OD 405 nm) was plotted against the HuMab concentrations and the curves were fitted using nonlinear regression. Best fit values for maximum binding (Bmax) are listed in Table 4, as are the correlation coefficient of the curve (R2) and the standard deviation for each value. The lowest correlation coefficient had a value of 0.950 (IgG4). With a maximum binding of 0.279, human IgG4 (negative control) shows minimum binding of C1q. Positive controls IgG1 and IgG3 both bind C1q, as shown by a maximum binding of 1.729 and 2.223, respectively.

Table 4:

Maximum binding (Bmax) of the HuMab tested in the C1q binding ELISA (n=3)

Best fit values	Bmax	Standard deviation Bmax	Goodness of fit R^2	Standard deviation R^2
IgG1	1.729	0.166	0.983	0.010
IgG3	2.223	0.947	0.995	0.005
IgG4	0.279	0.280	0.950	0.041
Antibody 1A	1.670	0.601	0.988	0.005
Antibody 8	1.954	0.131	0.978	0.009
Antibody 23	1.872	0.558	0.990	0.004

The correlation coefficient (R2) and standard deviation and are also listed.

Compared to the C1q binding of human IgG4 (negative control, with an O.D. of 0.279), all antibodies tested are equally capable of fixing C1q.

Example 15

Determination of antibody mediated effector functions by anti-IGF-IR HuMabs

In order to determine the capacity of the generated HuMab antibodies to elicit immune effector mechanisms, complement dependent cytotoxicity (CDC) and antibody-dependent cell cytotoxicity (ADCC) studies were performed.

To study CDC (National Cancer Institute, lung adenocarcinoma cell line), H322M, H460 and NIH 3T3 cells (2-6 x 10^6) were labeled with 100 µCi ^{51}Cr for 45-120 minutes (Amersham Pharmacia Biotech, UK, Cat CJS11). After labeling the cells were washed twice with 40 ml PBS and spun for 3 minutes at 1500 rpm. The cells were then plated 5,000 per well in a round bottom plate, in a volume of 50 µl. Antibodies were added at a final concentration ranging from 25-0.1 µg/ml in a volume of 50 µl cell culture medium to 50 µl cell suspension and incubated for 30-60 minutes. After incubation excess antibody was removed by washing twice with PBS. 100 µl of active or inactive (30 minutes at 56°C) pooled human serum, guinea pig, rabbit or nude mice serum diluted between 1/3-1/30 was added, and the cells were incubated for 3 hours, after which the cells were spun down at 1500 rpm for 3 minutes. 100 µl of the supernatant was harvested, transferred to polypropylene tubes and counted in a γ-counter.

To study the effects of the antibodies in ADCC, H322M, H460 and NIH 3T3 or other suitable IGF-IR expressing cells (2-6 x 10^6) were labeled with 100 µCi ^{51}Cr for 45-120 minutes (Amersham Pharmacia Biotech, UK, Cat CJS11), washed twice with 40 ml of PBS and spun for 3 minutes at 1500 rpm. The cells were plated 5,000 per well in a round bottom plate, in a volume of 50 µl. HuMab antibodies were added at a final concentration ranging from 25-0.1 µg/ml in a volume of 50 µl cell culture medium to 50 µl cell suspension and incubated for 15 minutes. Subsequently, 50 µl of effector cells, freshly isolated PBMC or purified effector cells from buffycoats, were added at an E:T ratio in the range of from 100:1 to 5:1. The plates were centrifuged for 2 minutes at 500-700 rpm, and incubated overnight at 37°C. After incubation the cells were spun down for 3 minutes at 1500 rpm and 100 µl of supernatant was harvested, transferred to polypropylene tubes and counted in a γ-counter.

The magnitude of cell lysis by CDC or ADCC is expressed as % of the maximum release of radioactivity from the target cells lysed by detergent corrected for spontaneous release of radioactivity from the respective target cells.

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Patent Claims

1. Antibody binding to IGF-IR and inhibiting the binding of IGF-I and IGF-II to IGF-IR, characterized in that said antibody

   a) is of IgG1 isotype and
   b) shows a ratio of inhibition of the binding of IGF-I to IGF-IR to the inhibition of binding of IGF-II to IGF-IR of 1:3 to 3:1 and
   c) induces cell death of 20% or more cells of a preparation of IGF-IR expressing cells after 24 hours at a concentration of said antibody of 100 nM by ADCC.

2. Antibody according to claim 1, characterized in that said antibody induces death of 20% or more cells of a preparation of IGF-IR expressing cells after 4 h at an antibody concentration of said antibody of 100 nM by CDC.

3. Antibody according to claim 1 or 2, characterized by being a human or humanized antibody.

4. Antibody according to claims 1 to 3, characterized by an affinity of about 10^{-11} to 10^{-8} M (K_D).

5. Antibody according to claims 1 to 4, characterized by comprising as complementarity determining regions (CDRs) having the following sequences:

   a) an antibody heavy chain comprising as CDRs CDR1 (aa 31-35), CDR2 (aa 50-66) and CDR3 (aa 98-108) of SEQ ID NO:1, wherein amino acid 31 can be asparagine or serine, amino acid 66 can be glycine or deleted, and amino acid 104 can be glutamic acid or aspartic acid;
   b) an antibody light chain comprising as CDRs CDR1 (aa 18-34 or aa 24-34), CDR2 (aa 50-56) and CDR3 (aa 89-98) of SEQ ID NO:2, wherein amino acid 96 can be proline or isoleucine, and amino acid 98 can be phenylalanine or deleted.

6. Antibody according to claims 1 to 5, characterized by

a) a heavy chain consisting of a variable region (VH) of SEQ ID NO:1, wherein amino acid (aa) 30 denotes serine or arginine, aa 31 denotes asparagine or serine, aa 94 denotes histidine or tyrosine and aa 104 denotes aspartic acid or glutamic acid, and of a human heavy chain constant region (CH);

b) and a light chain consisting of a variable region (VL) of SEQ ID NO:2, wherein aa 96 denotes proline or isoleucine, aa 100 denotes proline or glutamine, aa 103 denotes arginine or lysine, aa 104 denotes valine or leucine and aa 105 denotes aspartic acid or glutamic acid, and of a human light chain constant region (CL).

7. Antibody according to claim6, characterized in that in the heavy chain the amino acids 30, 31, 94 and 104 are

a) aa 30 Arg, aa 31 Asn, aa 94 Tyr and aa 104 Asp, or
b) aa 30 Arg, aa 31 Ser, aa 94 Tyr and aa 104 Asp, or
c) aa 30 Ser, aa 31 Asn, aa 94 His and aa 104 Glu.

8. Antibody according to claim 6, characterized in that in the light chain the amino acids 96, 100, 103, 104 and 105 are

a) aa 96 Pro, aa 100 Pro, aa 103 Lys, aa 104 Val and aa 105 Asp, or
b) aa 96 Ile, aa 100 Gln, aa 103 Arg, aa 104 Leu and aa 105 Glu.

9. Antibody according to claims 1 to 8 obtainable from hybridoma cell line <IGF-1R> HuMab Clone 1a, <IGF-1R> HuMab Clone 23 or <IGF-1R HuMab Clone 8.

10. The use of an antibody according to claims 1 to 9 for the manufacture of a pharmaceutical composition.

11. A pharmaceutical composition containing an antibody according to claims 1 to 9 in a pharmaceutically effective amount.

12. Hybridoma cell lines <IGF-1R> HuMab Clone 1a, <IGF-1R> HuMab Clone 23 and <IGF-1R HuMab Clone 8.

13. Method for the manufacture of a pharmaceutical composition comprising a pharmaceutically effective amount of an antibody according to claims 1 to 9.

14. A nucleic acid encoding a polypeptide capable of assembling together with the respectively other antibody chain defined below, whereas said polypeptide is either

a) an antibody heavy chain comprising as CDRs CDR1 (aa 31-35), CDR2 (aa 50-66) and CDR3 (aa 98-108) of SEQ ID NO:1, wherein amino acid 31 can be asparagine or serine, amino acid 66 can be glycine or deleted, and amino acid 104 can be glutamic acid or aspartic acid;

b) an antibody light chain comprising as CDRs CDR1 (aa 18-34 or aa 24-34), CDR2 (aa 50-56) and CDR3 (aa 89-98) of SEQ ID NO:2, wherein amino acid 96 can be proline or isoleucine, and amino acid 98 can be phenylalanine or deleted.

15. A nucleic acid according to claim 14, wherein said polypeptide is either

a) a heavy chain consisting of a variable region (VH) of SEQ ID NO:1, wherein amino acid (aa) 30 denotes serine or arginine, aa 31 denotes asparagine or serine, aa 94 denotes histidine or tyrosine and aa 104 denotes aspartic acid or glutamic acid, and of a human heavy chain constant region (CH);

b) and a light chain consisting of a variable region (VL) of SEQ ID NO:2, wherein aa 100 denotes proline or glutamine, aa 103 denotes arginine or lysine, aa 104 denotes valine or leucine and aa 105 denotes aspartic acid or glutamic acid, and of a human light chain constant region (CL).

16. An expression vector comprising a nucleic acid according to claim 14 or 15, capable of expressing said nucleic acid in a prokaryotic or eukaryotic host cell.

17. A prokaryotic or eukaryotic host cell comprising the vector according to claim 16.

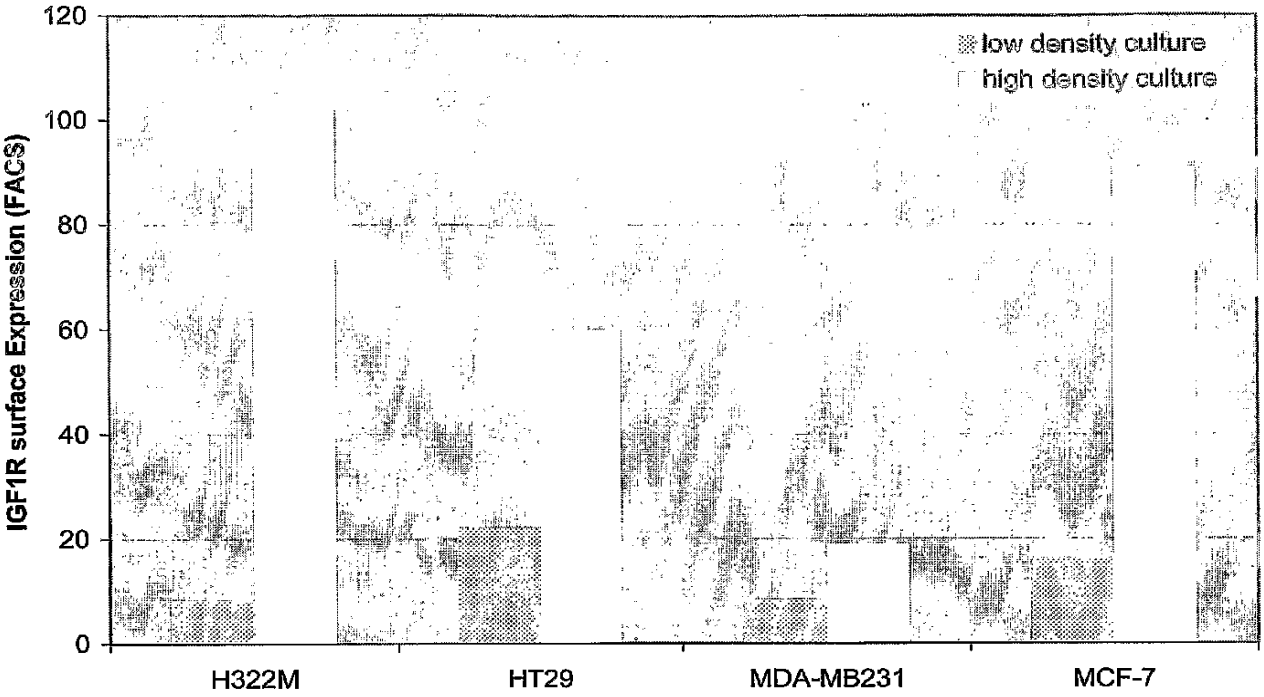
18. A method for the production of a polypeptide binding to IGF-IR and inhibiting the binding of IGF-I and IGF-II to IGF-IR, characterized by

expressing a nucleic acid encoding a heavy chain and a nucleic acid encoding a light chain according to claim 14 or 15 in a prokaryotic or eukaryotic host cell and recovering said polypeptide from said cell.

19. Method for the treatment of a patient in need of an antitumor therapy, characterized by administering to the patient a therapeutically effective amount of an antibody according to claims 1 to 9.

20. Method according to claim 19, characterized in that the antibody is administered in combination with a cytotoxic agent, a prodrug thereof or a cytotoxic radiotherapy.

Fig. 1


IGF1R surface Expression (FACS)
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100
80
60
40
20
0
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high density culture
H322M
HT29
MDA-MB231
MCF-7

Fig. 2

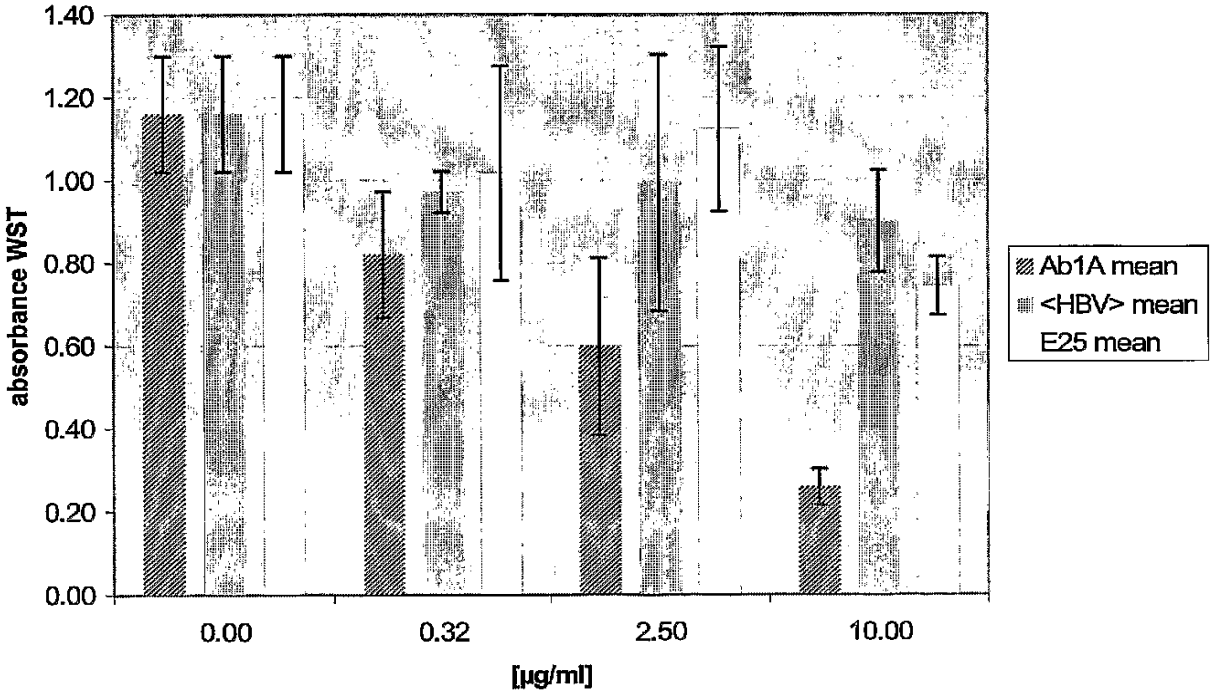

absorbance WST
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.00
0.32
2.50
10.00
[µg/ml]
Ab1A mean
<HBV> mean
E25 mean

Fig. 3

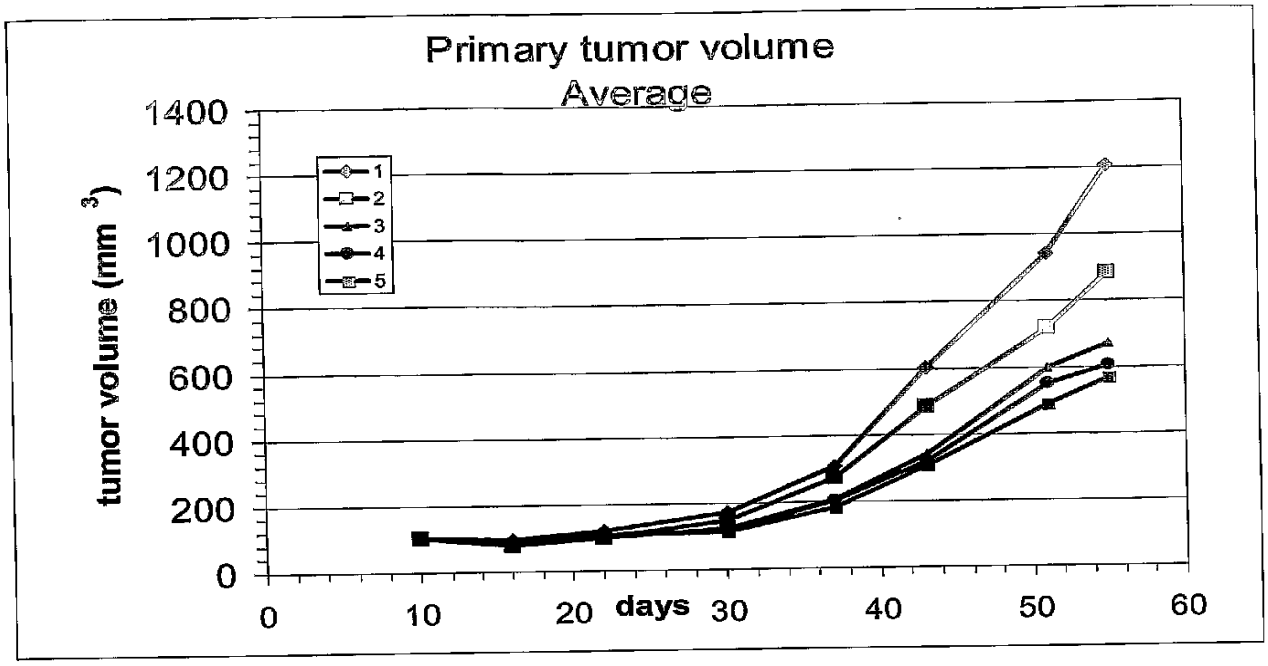

Primary tumor volume
Average
tumor volume (mm 3)
1400
1200
1000
800
600
400
200
0
1
2
3
4
5
0
10
20
days
30
40
50
60

Fig. 4

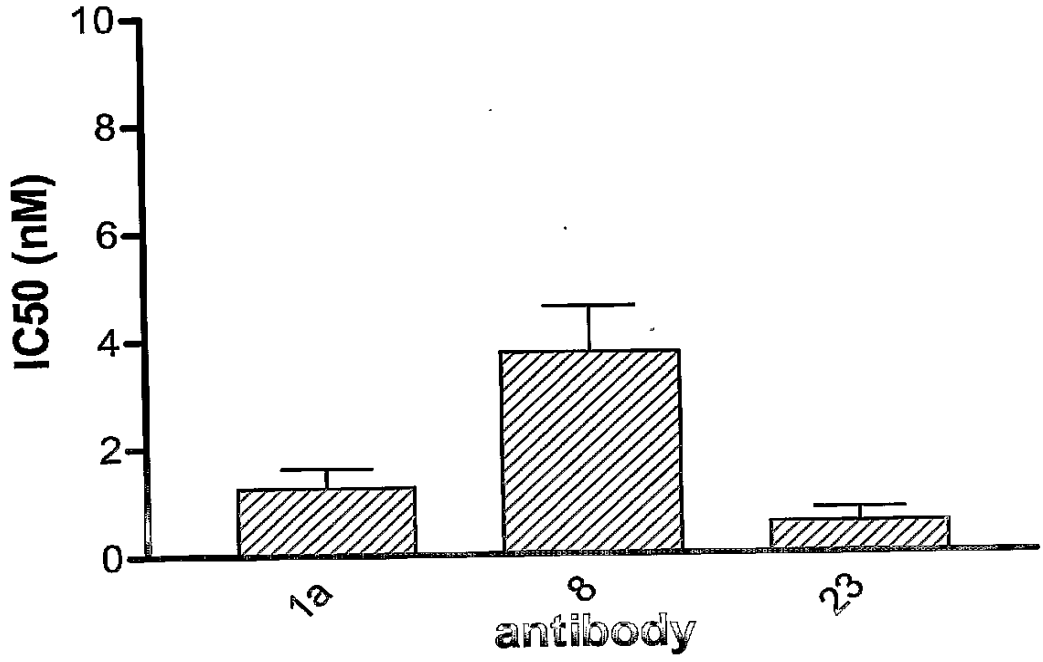
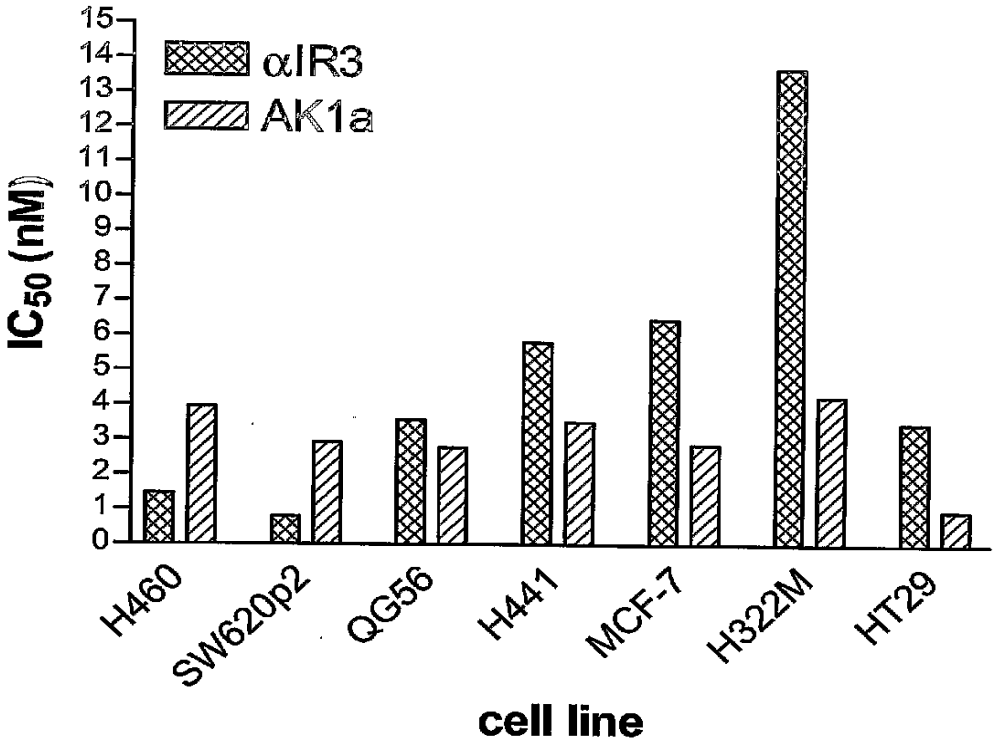

IC50 (nM)
10
8
6
4
2
0
1a
8
23
antibody

Fig. 5


αIR3
AK1a
IC50 (nM)
0
1
2
3
4
5
6
7
8
9
10
11
12
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14
15
H460
SW620p2
QG56
H441
MCF-7
H322M
HT29
cell line

Fig. 6

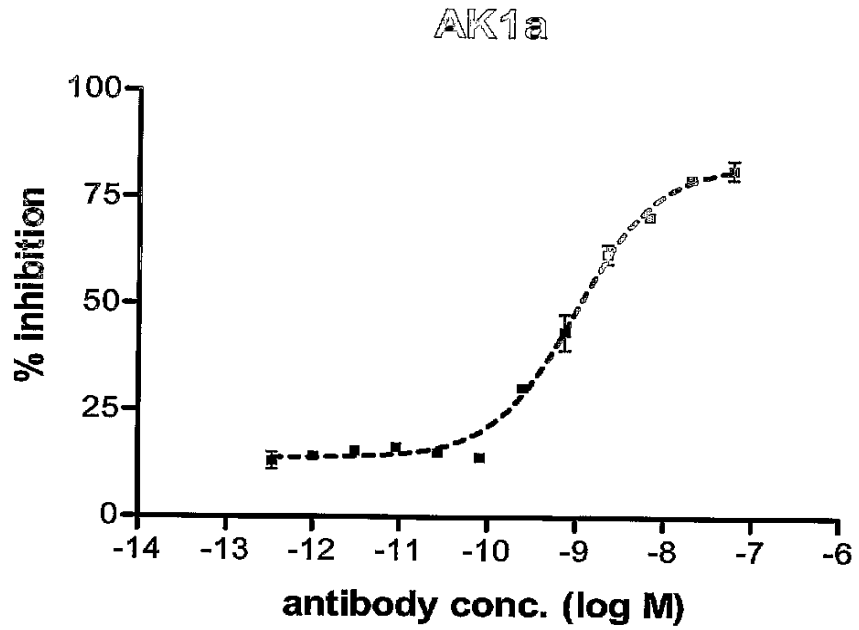

AK1a
100
75
50
25
0
% inhibition
-14
-13
-12
-11
-10
-9
-8
-7
-6
antibody conc. (log M)

Fig. 7

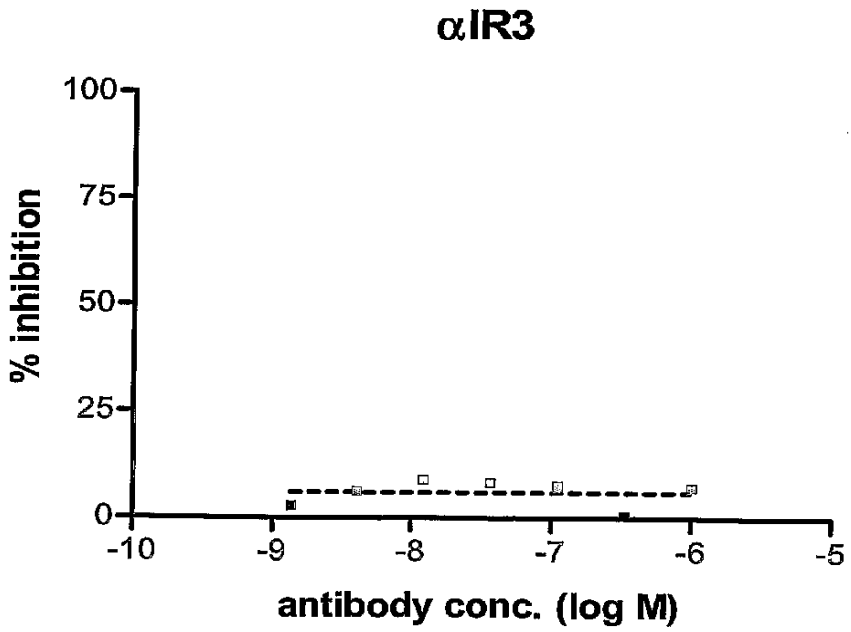
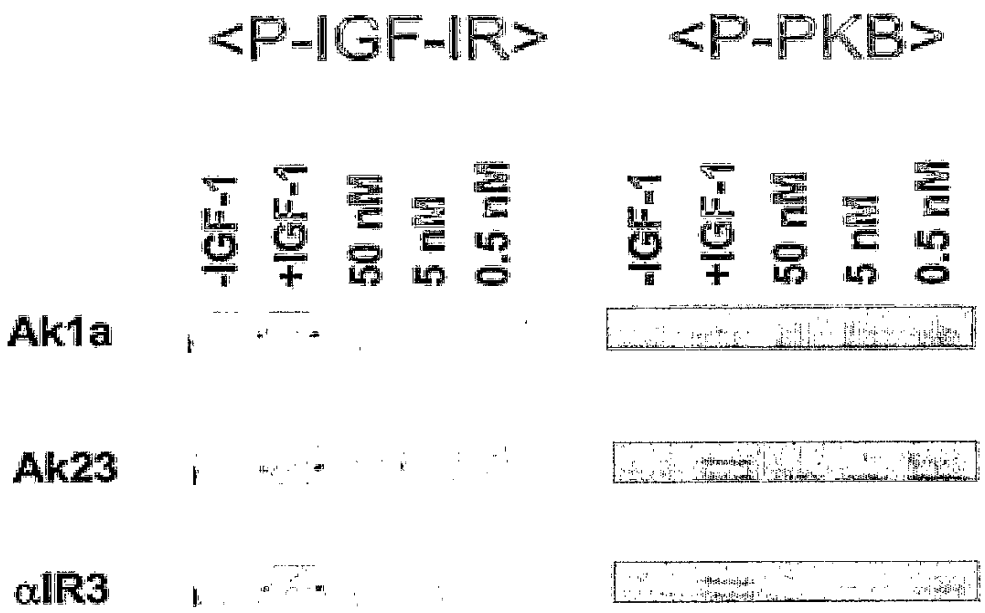

αIR3
100
75
50
25
0
% inhibition
-10
-9
-8
-7
-6
-5
antibody conc. (log M)

Fig. 8


<P-IGF-IR>
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-IGF-1
+IGF-1
50 nM
5 nM
0.5 nM
-IGF-1
+IGF-1
50 nM
5 nM
0.5 nM
Ak1a
Ak23
αIR3

Fig. 9

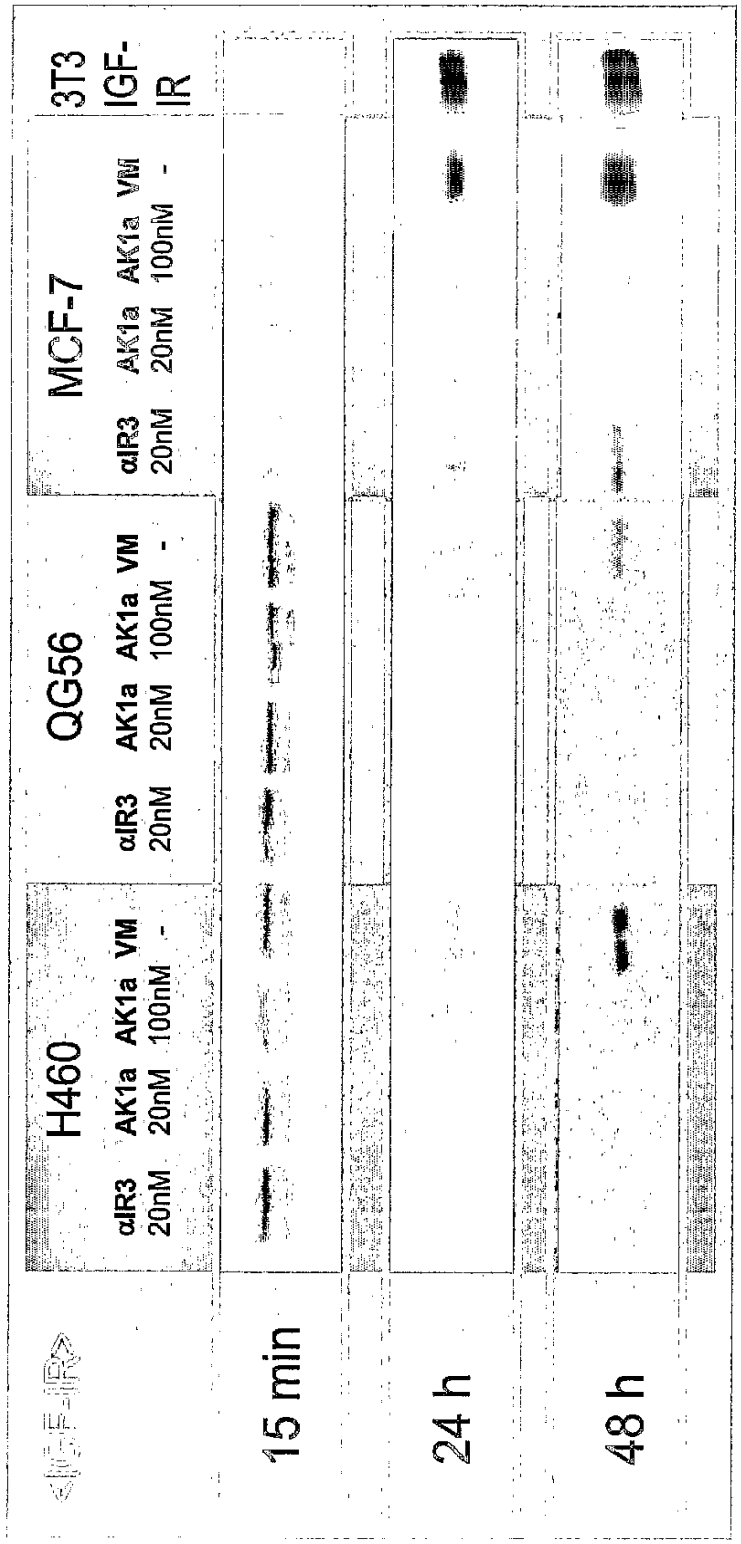

H460
αIR3 20nM
AK1a 20nM
AK1a 100nM
VM -
QG56
αIR3 20nM
AK1a 20nM
AK1a 100nM
VM -
MCF-7
αIR3 20nM
AK1a 20nM
AK1a 100nM
VM -
3T3 IGF-IR
<IGF-IR>
15 min
24 h
48 h

Fig. 10

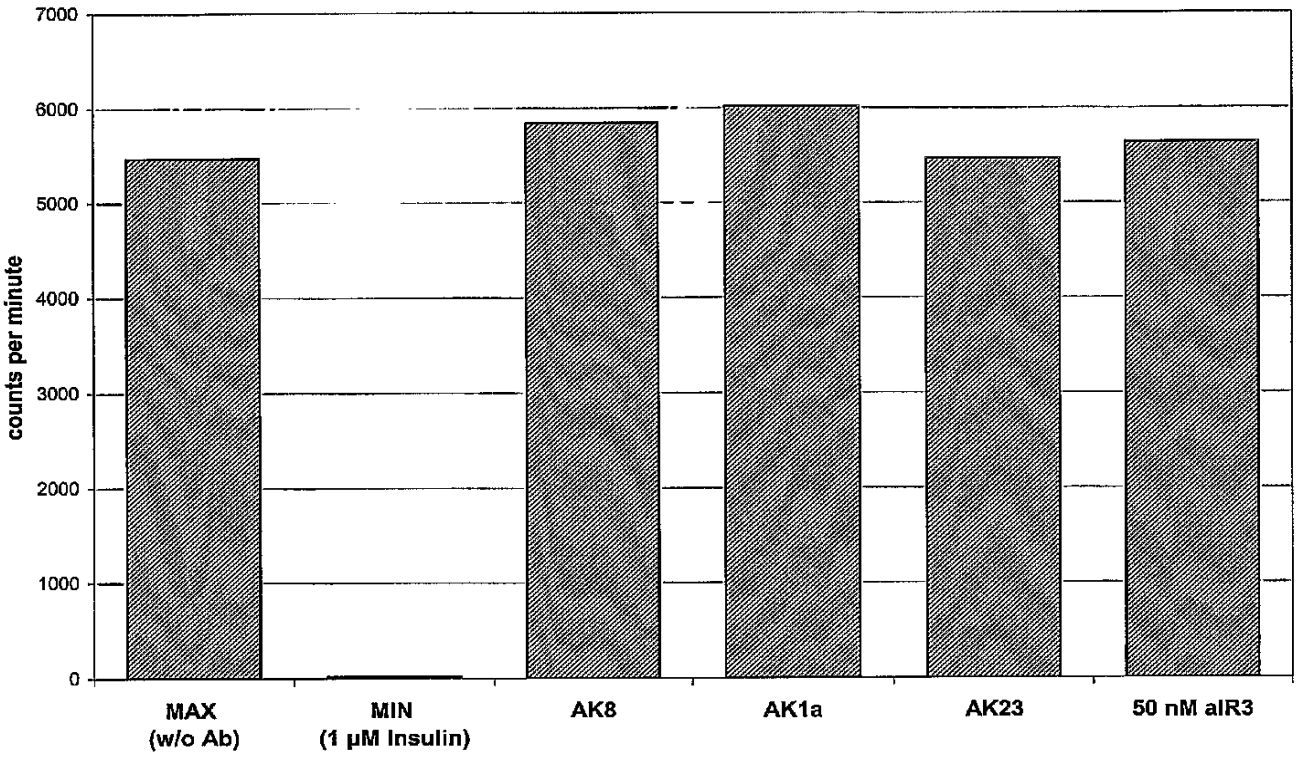
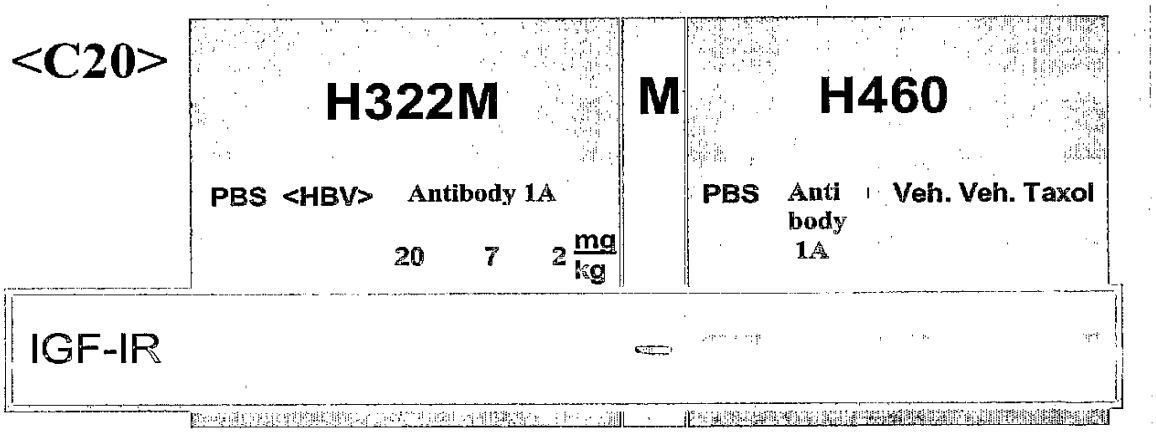

7000
6000
5000
4000
3000
2000
1000
0
counts per minute
MAX (w/o Ab)
MIN (1 µM Insulin)
AK8
AK1a
AK23
50 nM aIR3

Fig. 11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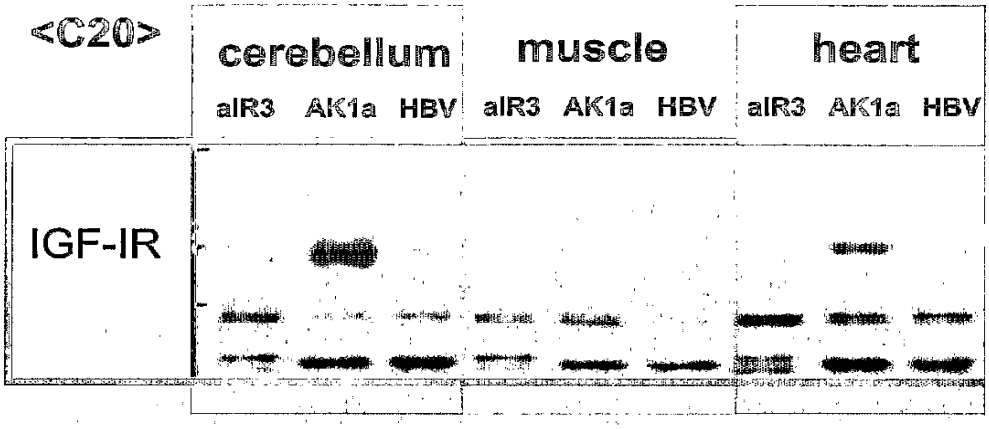
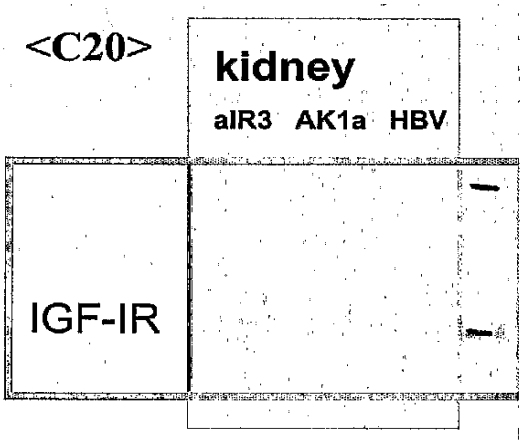
H322M
M
H460
PBS
<HBV>
Antibody 1A
20
7
2 mg/kg
PBS
Anti body 1A
Veh.
Veh.
Taxol
IGF-IR

Fig. 12

marmoset tissue



Cynomolgus cells



SEQUENCE LISTING

<110> F. Hoffmann-La Roche AG

<120> Antibodies against insulin-like growth factor I receptor and uses thereof

<130> 21655 WO

<150> US 60/459,837
<151> 2003-04-02

<150> US 60/463,003
<151> 2003-04-15

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35 40 45

Ser Ala Ile Gly Ser Gly Gly Gly Thr Tyr Tyr Ala Asp Ser Val Lys
50 55 60

Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr Leu
65 70 75 80

Gln Met Asn Ser Leu Arg Ala Glu Asp Met Ala Val Tyr Tyr Cys Ala
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Thr Met Val Thr Val Ser Ser
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35 40 45

Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
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Thr Phe Gly Pro Gly Thr Lys Val Asp Ile Lys
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20 25 30

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35 40 45

Ser Ala Ile Gly Ser Gly Gly Gly Thr Tyr Tyr Ala Asp Ser Val Lys
50 55 60

Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr Leu
65 70 75 80

Gln Met Asn Ser Leu Arg Ala Glu Asp Met Ala Val Tyr Tyr Cys Ala
85 90 95

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Thr Met Val Thr Val Ser Ser
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35 40 45

Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
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Thr Phe Gly Pro Gly Thr Lys Val Asp Ile Lys
100 105

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            20                  25                  30

Ala Met Tyr Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ser Ala Ile Gly Ser Gly Gly Gly Thr Tyr Tyr Ala Asp Ser Val Lys
    50                  55                  60

Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr Leu
65                  70                  75                  80

Gln Met Asn Ser Leu Arg Ala Glu Asp Met Ala Val Tyr His Cys Ala
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Thr Met Val Thr Val Ser Ser
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            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Glu Lys Ala Pro Lys Ser Leu Ile
        35                  40                  45

Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60
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Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

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1               5                   10                  15

agc acc tct ggg ggc aca gcg gcc ctg ggc tgc ctg gtc aag gac tac        96
Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr
            20                  25                  30

ttc ccc gaa ccg gtg acg gtg tcg tgg aac tca ggc gcc ctg acc agc       144
Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
        35                  40                  45

ggc gtg cac acc ttc ccg gct gtc cta cag tcc tca gga ctc tac tcc       192
Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
    50                  55                  60

ctc agc agc gtg gtg acc gtg ccc tcc agc agc ttg ggc acc cag acc       240
Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr
65                  70                  75                  80

tac atc tgc aac gtg aat cac aag ccc agc aac acc aag gtg gac aag       288
Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys
                85                  90                  95

aaa gtt gag ccc aaa tct tgt gac aaa act cac aca tgc cca ccg tgc       336
Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys
            100                 105                 110

cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc ccc cca       384
Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
        115                 120                 125

aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag gtc aca tgc       432
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
    130                 135                 140
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gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag ttc aac tgg      480
Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
145                 150                 155                 160

tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg gag      528
Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
                165                 170                 175

gag cag tac aac agc acg tac cgt gtg gtc agc gtc ctc acc gtc ctg      576
Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
            180                 185                 190

cac cag gac tgg ctg aat ggc aag gag tac aag tgc aag gtc tcc aac      624
His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
        195                 200                 205

aaa gcc ctc cca gcc ccc atc gag aaa acc atc tcc aaa gcc aaa ggg      672
Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
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cag ccc cga gaa cca cag gtg tac acc ctg ccc cca tcc cgg gat gag      720
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
225                 230                 235                 240

ctg acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa ggc ttc tat      768
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
                245                 250                 255

ccc agc gac atc gcc gtg gag tgg gag agc aat ggg cag ccg gag aac      816
Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
            260                 265                 270

aac tac aag acc acg cct ccc gtg ctg gac tcc gac ggc tcc ttc ttc      864
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
        275                 280                 285

ctc tac agc aag ctc acc gtg gac aag agc agg tgg cag cag ggg aac      912
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
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Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
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Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
50 55 60

Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr
65 70 75 80

Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys
85 90 95

Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys
100 105 110

Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
115 120 125

Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
130 135 140

Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
145 150 155 160

Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
165 170 175

Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
180 185 190

His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
195 200 205

Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
210 215 220

Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
225 230 235 240

Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
245 250 255

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Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
            260                 265                 270

Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
        275                 280                 285

Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
    290                 295                 300

Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
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Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
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1               5                   10                  15

cag ttg aaa tct gga act gcc tct gtt gtg tgc ctg ctg aat aac ttc        96
Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe
            20                  25                  30

tat ccc aga gag gcc aaa gta cag tgg aag gtg gat aac gcc ctc caa       144
Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln
        35                  40                  45

tcg ggt aac tca cag gag agc gtc aca gag cag gac agc aag gac agc       192
Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser
    50                  55                  60

acc tac agc ctc agc agc acc ctg acg ctg agc aaa gca gac tac gag       240
Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
65                  70                  75                  80

aaa cac aaa gtc tac gcc tgc gaa gtc acc cat cag ggc ctg agc tcg       288
Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser
                85                  90                  95
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ccc gtc aca aag agc ttc aac agg gga gag tgt                          321
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
            100                 105
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            20                  25                  30

Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln
        35                  40                  45

Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser
    50                  55                  60

Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
65                  70                  75                  80

Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser
                85                  90                  95

Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
            100                 105
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Applicant's or agent's file reference	International application No.
21655 WO-SR	PCT/EP2004/003442

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 6, line table.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1b
D-38124 Braunschweig

Date of deposit	Accession Number
10.04.2003 / 24.04.2003*	DSM ACC2586, DSM ACC2588 / DSM ACC 2589*

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☒

Indications relating to the Expert Solution in respect of the deposited biological material DSM ACC2586, DSM ACC 2588 and DSM ACC 2589

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

CA (Canada), EP (European Patent), SG (Singapore)

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☒ This sheet was received by the International Bureau on: 25 JUNE 2004 (fax)
Authorized officer	Authorized officer EKO

C. ADDITIONAL INDICATIONS (additional sheet)

Applicants	:	F. Hoffmann-La Roche AG, et al.
Applicants file reference	:	21655 WO-SR
International application No.	:	PCT/EP2004/003442

Indications Relating to the Expert Solution in Respect of Deposited Biological Material Referred to in the Description

The indications relating to deposited biological material are all contained in the description. The following additional indications are not required to be part of the description and should be treated as "separate indications." They relate only to the expert solution.

The additional indications made below relate to the deposited biological material referred to as:

<IGF-1R>HUMAB-Clone 1a	DSM ACC2586
<IGF-1R>HUMAB-Clone 23	DSM ACC2588
<IGF-1R>HUMAB-Clone 8	DSM ACC2589

in the description on page 6.

The additional indications are:

For CA (Canada) designation:

In respect of the designation of Canada, samples of the deposited biological material will be made available until the grant of the Canadian patent, or until the date on which the application is refused, or is abandoned and no longer subject to reinstatement, or is withdrawn, as provided in Rules 107 and 108 of the Patent Rules under the Canadian Patent Act, only by the issue of a sample to an independent expert nominated by the Commissioner (Rule 104(4))

For EP (European Patent) designation:

In respect of the designation of the EPO, samples of the deposited biological material will be made available until the publication of the mention of the grant of the European patent, or until 20 years from the date of filing if the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC, only by the issue of a sample to an expert nominated by requester (Rule 28(4) EPC).

For SG (Singapore) designation:

Applicants hereby give notice of our intention that samples of the above-identified culture shall be available only to experts in accordance with paragraph 3 of the Fourth Schedule to the Patents Rules 1995.